The Use of Novel Techniques to Study the Roles of Cytokines in Joint Pain and Inflammation

By

Nicola J. Barton BSc (Hons.)

Doctor of Philosophy
University of Edinburgh
2007

DECLARATION

I declare that this thesis was composed entirely by myself and represents all my own work except for the procedures listed below and acknowledged in the text.

1. Histological samples were processed, paraffin embedded, sectioned and stained by Professor Donald Salter (The department of Pathology, The University of Edinburgh, UK).
2. The Pressure Application Device was designed and constructed by Dr Harry Brash (Department of Hepatology, The University of Edinburgh, UK).

ACKNOWLEDGEMENTS

There are numerous people I would like to thank for their help, guidance and support over the last three years. Firstly I would like to thank my supervisors, Prof. Danny McQueen, Dr Alison Reeve and Dr Alex Wilson for all their support and encouragement, not to mention having more faith than I did that I was a suitable PhD candidate to begin with! Thanks to GlaxoSmithKline for providing the funding for the PhD project and allowing me to use resources and equipment on site when necessary.

I would also like to thank Prof. Donald Salter for his help with the histological processing and evaluation, as well as taking the time to explain everything to me and answer my questions. In addition I would like to acknowledge a few people who taught me new techniques and then took the time to read subsequent manuscripts, adding their invaluable comments: Jane Hughes and Dave Stevens at GSK, who spent time teaching me to use the Luminex system and Prof. Adriano Rossi and Debbie Sawatzky for their tuition and time with my ELISA and cell counting assays. A special thanks to Mrs Susan Bond for all her help in the lab, for proof reading this thesis, a task I did not envy, and one I suspect she regretted as soon as she volunteered! She has also been precious company over the years and particularly during several long behavioural trials!

Finally, I would like to say thank you to all my family, in particular my Mum and Dad for always supporting me, I will get a proper job soon I promise! Thanks to some good friends, Catriona, James, Helen, Craig and Iain for their very welcome non-science chat and many a good night out! In particular a huge thanks to Matt for his love and support (not to mention cleaning and cooking duties over the last few months) without which I may never have made it to this point!!

ASSOCIATED PUBLICATIONS

The publications (full papers and abstracts) arising from this thesis are listed below:

N.J. Barton, D.S. McQueen, S.D. Gauldie, A.W. Wilson, N.M.Clayton and I.P. Chessell (2004) Mice lacking the TRPV1 receptor develop milder joint inflammation following FCA. *Poster presentation at The British Pharmacological Society, Winter Meeting, Newcastle, UK. British Journal of Pharmacology*; pA_2 online.

N.J. Barton, D.S. McQueen, S.D. Gauldie, A.W. Wilson, N.M.Clayton and I.P. Chessell (2005) The relevance of the chilli pepper receptor in arthritis. *Poster presentation at SET for Britain, Bioscience event, The House of Commons, London, UK*

N.J. Barton, I.T. Strickland, A.J. Reeve, I.P. Chessell and D.S. McQueen (2006) IL1β or IL6 induces mechanical hypersensitivity and allodynia in the rat knee joint. *Presented as a poster and chosen as 3rd prize in Poster Prize Presentation (oral) Award.*

I.T.Strickland, N.J.Barton, H.M.Brash, D.S.McQueen, A.J.Reeve, A.W.Wilson and I.P.Chessell (2005) A novel behavioural readout for assessing hypersensitivity of knee joints in a murine model of unilateral arthritis. *Poster presentation at the British Pharmacological Society, Winter Meeting, London, UK. British Journal of Pharmacology, pA2 online.*.

N.J. Barton, D.S. McQueen, D. Thomson, S.D. Gauldie, A.W. Wilson, D.M. Salter and I.P. Chessell (2006) Attenuation of experimental arthritis in TRPV1R knockout mice. Experimental and Molecular Pathology, 81 (2), p166-170

N.J. Barton, I.T. Strickland, S.M. Bond, H.M. Brash, A.J. Reeve, S.T. Bate, A.W. Wilson, I.P. Chessell and D.S. McQueen (2007) Pressure Application Device (PAD): A novel behavioural technique for measuring hypersensitivity in rodent models of joint pain. The Journal of Neuroscience Methods, 163, p67-75.

N.J. Barton, D.A. Stevens, J.P. Hughes, A.J. Reeve, A.G. Rossi, P.C. Staton, I.P. Chessell and D.S. McQueen (2007) Demonstration of a novel technique to assess inflammatory mediator and cell content from synovial fluid of rat knee joints. The Journal of Inflammation, 4 (13).

ABSTRACT

Rheumatoid arthritis (RA) is a common, chronic, autoimmune, inflammatory disease characterized by persistent synovitis that results in the progressive destruction of joints. The cellular and molecular basis of the inflammation is complex and multifactorial. During the progression of the disease many types of cells are activated, which in turn secrete a variety of mediators, including cytokines, which initiate and perpetuate the disease.

Rat adjuvant-induced unilateral arthritis is a well established RA disease model and use of this model has facilitated the understanding of the pathology of joint inflammation. The model closely mimics the pathology of human RA, including histopathological changes, cell infiltration, as well as hypersensitivity and swelling of the joint. Measurements of spontaneous pain and hypersensitivity states are assessed in this model. However, no objective measure of joint hypersensitivity is used to assess experimental arthritic joint pain in laboratory rodents. To that end, the pressure application device (PAD) was developed to align pre-clinical measures to those used clinically and help the translation of animal studies to human conditions. PAD was able to detect FCA-induced hypersensitivity in mice and rats, observed as a decrease in limb withdrawal thresholds (LWTs) of around 60% and 40% respectively, compared with basal levels in normal joints. PAD subsequently detected prednisolone analgesia in both species, which was abolished after dosing ceased. PAD also showed significant reversal of evoked mechanical hypersensitivity in arthritic animals treated with morphine or celecoxib, which was comparable to that measured by the weight distribution readout. PAD provides a novel, accurate behavioural tool for detecting localised primary mechanical hypersensitivity in two animal models of chronic inflammatory joint pain.

The infiltration of cells and release of inflammatory proteins in the synovial tissue and joint space is a key characteristic of synovitis. Measuring the levels of these in the synovial fluid can provide information about the underlying pathophysiology of joint

disease. Furthermore changes occurring in the synovial fluid can be used as biomarkers of disease; therefore the joint perfusion method was developed to evaluate the inflammatory protein and cell content of rat knee joints, to further validate the adjuvant-induced arthritis model, as well as to determine the effects of inflammatory insults or the effect of anti-inflammatory, analgesic or anti-rheumatic drugs. This technique proved to be reliable and consistent when perfusing the joint cavity, and regular volumes of sample were easily collected. This technique is therefore a valuable addition to protocols which use homogenates of entire joints to assess inflammatory mediator content.

The temporal expression patterns of cytokines and inflammatory cells in the knee joints of rats following induction of arthritis were determined using the novel perfusion technique. Cytokine expression altered over time as arthritis progressed from the acute to the more "chronic" phase. The proportion of inflamed joints that contained detectable levels of each mediator measured was significantly increased during the study. This suggests that it may be the presence of the protein, even at low levels, that is important for the development and maintenance of joint inflammation and hypersensitivity. In addition, significant correlations between measures of joint swelling or mechanical hypersensitivity and levels of cytokines in inflamed joints were seen. Prednisolone did not affect the absolute levels of cytokines in inflamed joints, although it reduced the percentage of inflamed joints that contained detectable levels of IL1α and IL6. This suggests that the steroid appears to have an all-or-none effect in terms of cytokine expression levels in this study.

The roles of IL1β and IL6 in joint pain and inflammation were assessed. The contribution of the activity of primary afferent fibres to joint pain and hypersensitivity after administration of intra-articular IL1β or IL6 was investigated by recording action potentials from primary afferent nerves innervating the knee joint. IL1β caused a transient increase in the frequency of basal neural discharge by 88% within three hours. It also decreased the threshold of mechanical stimulation required to evoke neural activity by 50% between one and four hours after injection. In contrast, IL6 did not affect the frequency of basal neural

discharge or the mechanical threshold. Neither IL1β nor IL6 affected the neural discharge frequency to mechanical stimulation above the threshold. The induction of basal neural activity resembles the occurrence of spontaneous pain during inflammation, such as that measured by the incapacitance tester as a result of intra-articular IL1β or IL6. A reduction in the LWT, measured by PAD, following IL1β or IL6 occurred within a few hours, similar to the decrease in the mechanical threshold to von Frey hairs in primary afferents after IL1β, as a result of neuronal sensitization. Although IL1β or IL6 did not cause swelling of the joint, they did induce mechanical hypersensitivity within a couple of hours, which lasted for up to four days. Intra-articular IL1β or IL6 had no effect on joint structure, bone or cartilage. IL1β and IL6 evoked increases in the expression of IL1β, IL6 and TNFα within the first eight hours, and additionally elevated levels of IL1α, IL2, IL4 (IL6-treated only) and IL10 (IL1β-treated only) from day one post administration. IL1β also resulted in recruitment of inflammatory cells into the synovial cavity one day after administration.

In conclusion, this study has developed and validated two novel techniques to study experimental joint pain and inflammation in rodents; the behavioural measure of joint mechanical hypersensitivity, PAD; and the joint perfusion technique to assess inflammatory mediator and cell content of synovial fluid. These methods have been used alongside other techniques to show the temporal cytokine expression patterns during adjuvant-induced arthritis and the relationship of these to swelling and hypersensitivity of the joint. The roles of IL1β and IL6 in evoking joint pain and hypersensitivity were also investigated. This data supports the hypothesis that IL1β and IL6 are directly involved in the development of joint pain, but cannot alone elicit swelling or joint damage at doses sufficient to evoke hypersensitivity. Furthermore, similarities between this animal model of joint disease and human RA have been demonstrated that further validate the model as a valuable pre-clinical tool to study the inflammatory process of human RA. Moreover, consolidation of these similarities helps improve the confidence of novel drug screening using this model prior to use in the clinic.

CONTENTS

List of Figures

List of tables

List of abbreviations

°C	degrees Celsius
5-HT	5-hydroxytriptamine
AC	adenyl cyclase
Ach	acetylcholine
ACR	American college of rheumatology
ANOVA	analysis of variance
APP	acute phase proteins
ASIC	acid sensing ion channels
ATP	adenosine triphospahte
AUC	area under the curve
BK	bradykinin
BP	blood pressure
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CB	cannabinoid
CED	Cambridge electronic design
CGRP	calcitonin gene related peptide
CIA	collagen-induced arthritis
CMR	cold menthol receptor
CNS	central nervous system
COX	cyclooxygenase
CPJ	cartilage pannus junction
CRP	c-reactive protein
DAG	diacylglycerol
dH_20	distilled water
DMARD	disease modifying anti-rheumatic drug
DRG	dorsal root ganglion
EDTA	ethylenediaminetetraacetic acid
FCA	Freunds complete adjuvant
gf	gram force
GM-CSF	granulocyte-macrophage colony stimulating factor
gp130	glycoprotein 130
GPCR	guanyl-nucleotide protein coupled receptor
HLP	heavy liquid paraffin
i.art	intra-articular
i.p.	intra-peritoneal
i.v.	intra-venous
IASP	International association for the study of pain
ICAM	inter-cellular adhesion molecule
ICE	interleukin converting enzyme
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL1 R I	interleukin 1 receptor type 1
IL1 Ra	interleukin 1 receptor antagonist
IL1 RAcP	interleukin 1 receptor accessory protein
IL1 RII	interleukin 1 receptor type 2
IL6R	interleukin 6 receptor
iNOS	inducible nitric oxide synthase
IP_3	inositol triphosphate
kD	kilo Dalton
LPS	lipopolysacchardide
LT	leukotriene
LWT	limb withdrawal threshold
MAN	medial articular nerve
MMP	matrix metalloproteinase's
mRNA	messenger ribonucleic acid
MscL	mechano-sensitive channel of large conductance
NGF	nerve growth factor
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
OD	outer diameter
OPG	osteoprotegrin
OPG-L	osteoprotegrin ligand
P	statistical probability
PAD	pressure application device
PAF	platelet activating factor
PAG	periaquaductal gray
PAN	posterior articular nerve
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PEG	polyethylene glycol
PG	prostaglandin
PGE_2	prostaglandin E_2
PGI_2	prostacyclin
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMN	polymorphonuclear leukocytes
Pt/Ir	platinum/ iridium
RA	rheumatoid arthritis
rpm	revolutions per minute
RVM	rostra ventromedial medulla
s.c.	subcutaneous
S.E.M.	standard error of the mean
SCW	streptococcal cell wall
sIL-6R	soluble interleukin 6 receptor
TGFβ	transforming growth factor β
TNF	tumour necrosis factor
TRP	transient receptor potential
TTX	tetrodotoxin
TX	thromboxane

Chapter 1. Introduction

This chapter will give an overview of historical and more recent work surrounding the main themes of this thesis, including the pathology of arthritis, the roles of immune cells and cytokines in this disease as well as commonly used experimental models of joint disease. In addition, central and peripheral mechanisms of inflammatory pain and nociception will be discussed alongside the roles of various mediators and receptors involved in these processes.

1.1 ARTHRITIS

Arthritis can be divided according to its primary pathology into rheumatoid arthritis (RA) and osteoarthritis (OA).

1.1.1 OSTEOARTHRITIS

OA is a slowly progressing degenerative disease that mainly affects synovial joints through non-immune processes. It is commonly associated with one joint, and is usually the result of "wear and tear". The main clinical symptoms are joint swelling, stiffness, reduced function and a decrease in quality of life due to persistent, chronic pain (Scott, 2006). In OA, cartilage of the affected joint gradually roughens and becomes thin, the bone underneath thickens and at the edge of the joint it grows outward (see Figure 1.1). The synovium produces extra fluid resulting in joint swelling. The capsule and ligaments slowly thicken and contract to stabilize the joint as it changes shape. Muscles around the joint weaken and become wasted. In severe OA, the cartilage can degrade completely, so it no longer covers the thickened bone ends causing them to touch and start to wear away. The loss of cartilage, the wearing of bone and the bony overgrowth at the edges can change the shape of the joint, forcing the bones out of their normal position and causing deformity (Swagerty & Hellinger, 2001).

1.1.2 RHEUMATOID ARTHRITIS

RA is a common chronic autoimmune, inflammatory disease characterized by persistent synovitis that results in progressive destruction of affected joints. Its prevalence within the general population of the UK is 1.16% in women and 0.44% in men (Symmons *et al.*, 2002), these figures show a decrease since 1961, when the last age- and sex- specific estimates of the prevalence of RA were published (Lawrence, 1961). The disease can occur at any age, but it is most common in those aged 40 - 70 years, its incidence increasing with age. Pain is the most prominent symptom associated with arthritis (Anderson *et al.*, 1994; Heiberg & Kvien, 2002; Kazis *et al.*, 1983; McKenna & Wright, 1985) and a common basis for primary care consultation (Mantyselka *et al.*, 2001; Rekola *et al.*, 1993; Uhlig *et al.*, 2002). From a random sample of 120 RA patients, 47% ranked pain relief as the most desirable objective of their treatment (Gibson & Clark, 1985).

The past decade has seen major transformations in the treatment of RA in terms of both approach and choice of drugs. Previously, initial management consisted mainly of non-steroidal anti-inflammatory drugs (NSAIDs), which reduce joint inflammation, with the hope of improving the associated pain; then disease modifying anti-rheumatic drugs (DMARDs) were prescribed once clear evidence of erosions were seen. More recently, DMARDs have been introduced to treatment regimes much earlier following diagnosis, and are used in combination therapy in patients with the potential for progressive disease (Lee & Weinblatt, 2001), as they not only improve inflammatory symptoms, but also slow the progression of the underlying pathology and joint destruction.

RA is a systemic inflammatory disease, it can affect many tissues and organs (Scott, 2006) and causes general ill health. However, it principally attacks the joints, producing a progressive synovitis that often progresses to destruction of the articular cartilage and erosion of the bone due to persistent inflammation of the joint (see Figure 1.1). RA is a polyarthritis and is usually present in multiple joints; in synovial joints the inflammation is

symmetrical, with joints on both sides of the body equally affected (Scott, 2006). Although the cause of RA remains unknown, autoimmunity appears to play a pivotal role in its chronicity and progression (Glynn, 1968).

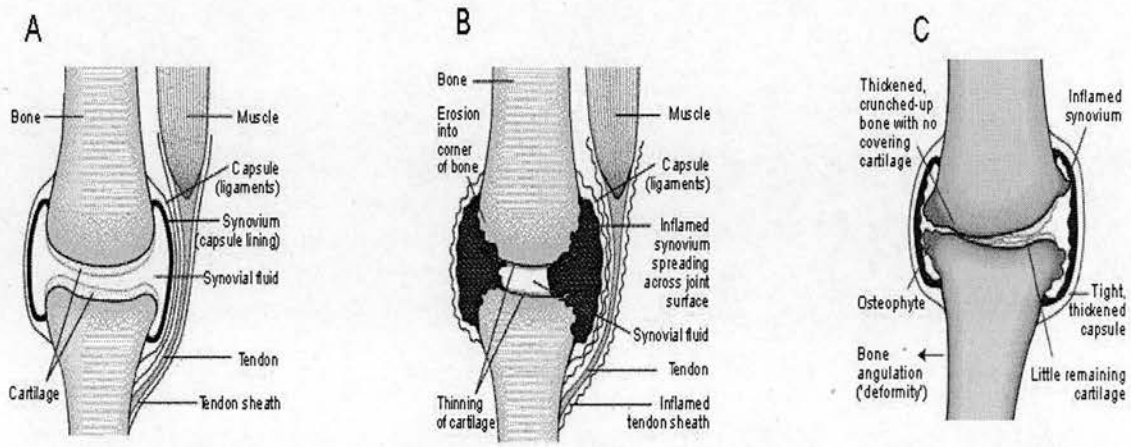


Figure 1.1 Comparison of a normal joint (A), a severe RA knee joint (B) and a severely deformed knee joint due to OA (C). The normal joint has a thin synovial lining and smooth surfaces on the bone and cartilage. The RA joint shows a marked thickening of the synovial membrane, due to the infiltration of inflammatory cells, which also invade surrounding tissues and the joint space. The principal features of OA include loss of articular cartilage, new bone formation in the subchondral region, and formation of new cartilage and bone at the joint margins (adapted from ArthritisResearchCampaign, 2004).

Anatomy of the knee joint and changes during RA

The **synovium** serves as a source of nutrients for cartilage as the cartilage itself is avascular (Gray, 1918). In addition, synovial cells (synoviocytes) synthesize joint lubricant such as hyaluronic acid, as well as collagens and fibronectin that constitute the structural framework of the synovial interstitum (Gray, 1918). In a normal joint the synovial lining consists of a very thin membrane, just a few cells thick, which isolates the largely acellular joint space (Bathon, 2005). During RA, synovitis or inflammation of the synovium occurs, involving a significant thickening of the synovial membrane as a result of a marked increase in macrophage-like and fibroblast-like synoviocytes as well as the invasion of inflammatory cells, mostly polymorphonuclear (PMN) leukocytes, into the synovial membrane and joint space (Bathon, 2005).

The **subintimal area** of synovium is where the synovial blood vessels are located; this area normally has very few cells. In RA, however, the subintimal area is infiltrated with

inflammatory cells, including T and B lymphocytes, macrophages and mast cells (Harris, 1990). This is accompanied by angiogenesis, the growth of new blood vessels, which supply nutrients and oxygen to the augmented inflammatory cell mass, thus contributing to the perpetuation of synovitis (Taylor & Feldmann, 2004). However, the new blood vessel network is dysfunctional and fails to restore tissue oxygen homeostasis, so the rheumatoid joint remains a hypoxic environment (Mapp *et al.*, 1995).

One of the characteristic features of RA is the formation of **pannus**, the tissue formed at the boundary of the cartilage and the synovial lining (Rosenberg, 1999). It is a fibrocellular mass of synovium consisting of both synoviocytes and inflammatory cells. The aggressive front of pannus overgrows healthy cartilage from the border with the synovium and destroys local articular structures. Within the pannus tissue, mediators are released, including neuropeptides, cytokines, oxygen radicals and lysosomal and matrix metalloproteinase (MMP) enzymes that perpetuate the inflammation and which cause cartilage destruction and bone erosion (Chu *et al.*, 1992). After the cartilage has been destroyed the pannus bridges the apposing bones and eventually ossifies (Rosenburg, 1999).

The joint **cartilage** consists of chondrocytes embedded in a highly structured matrix; it is a resilient tissue that absorbs considerable impact and stress. Chondrocytes are connective tissue cells that produce and maintain the cartilaginous matrix. In cartilage from RA patients, the collagen is impaired both structurally and functionally due to the production of proteolytic enzymes both by synovial lining cells and the chondrocytes themselves. During RA the synthesis of the matrix components are reduced and the breakdown of the cartilage matrix is enhanced. PMNs within the synovial fluid also contribute to cartilage breakdown (Bathon, 2005). In RA, the **bone** becomes eroded by the invading synovium via release of mediators such as prostaglandins (PGs) and proteases by the synovial cells and osteoclasts (multinucleated cells that degrade and reabsorb bone, which are involved in the natural turnover of bone tissue).

The synovial cavity is normally a space filled with hyaluronic acid and very few cells, however, in RA large amounts of fluid collect which are filtrates of plasma with high protein content. The synovial fluid is highly inflammatory in RA joints.

Cellular component of RA

During the course of RA, the normally acellular synovium becomes heavily infiltrated by a wide variety of cells which migrate from proliferating blood vessels. Variable numbers of T cells, B cells, plasma cells, macrophages, dendritic cells (antigen-presenting cells), fibroblasts, granulocytes and endothelial cells are dispersed throughout the synovium (Haraoui *et al.*, 1991; Harris, 1990; Muller-Ladner *et al.*, 1997; Tak, 2000), see Figure 1.2 and 1.3 for a summary.

After the initiating event of RA, an influx of CD4+ **T cells** into the synovium begins (Goronzy & Weyand, 1995). CD4 is a glycoprotein, part of the immunoglobulin (Ig) family, expressed on the surface of T helper cells, regulatory T cells and dendritic cells. On T cells, CD4 is the co-receptor for the T cell receptor; it amplifies the signal by the T cell receptor by recruiting tyrosine kinases essential for activating many molecules involved in the signalling cascade of an activated T cell. T cells recruited to the synovium are activated and release several cytokines, which activate monocytes, macrophages and fibroblasts. Although there appear to be large numbers of CD4+ T cells in the synovium throughout the disease course, they are mostly inactive in the chronic phase of the disease (Firestein & Zvaifler, 2002). This is shown by the fact that T-cell derived-cytokines are present in low levels (Smeets *et al.*, 1998) compared with macrophage and fibroblast-derived cytokines within the synovium (Firestein *et al.*, 1990). Moreover, experimental T-cell directed therapies have had limited success (Fox, 1997).

Fibroblast- and **macrophage-like cells** accumulate in the lining layer of the synovium (Barland *et al.*, 1962; Ghadially & Roy, 1966) where they produce large amounts of proinflammatory cytokines, chemokines and angiogenic molecules which activate signal

transduction pathways and transcription factors, which, in turn control the transcription of further pro- and anti-inflammatory cytokines (Sweeney & Firestein, 2004). This maintains the inflammatory cells in an activated state promoting the maturation of newly recruited monocytes and macrophages, resulting in a positive feedback cycle, perpetuating the inflammatory reaction.

Synovial tissue **macrophages** are multifunctional cells that have biological functions that may be either destructive or protective (Burmester *et al.*, 1997). When activated they demonstrate increased expression and transcription of interleukin (IL) 1β, tumour necrosis factor (TNF) α and the monocyte chemo-attractant protein. Macrophages and fibroblasts appear to be largely responsible for creating a self-perpetuating state of chronic inflammation in which continuing T cell participation may not be critical once the inflammatory reaction has begun (see Figure 1.3; Bathon, 2005).

In contrast to the rheumatoid synovial tissue of patients with RA, which is infiltrated with lymphocytes and macrophages, the predominant cell type in the synovial fluid is **neutrophils** (Chatham *et al.*, 1993). Neutrophils are recruited in large numbers to the rheumatoid synovium via expression of adhesion molecules on endothelial cells. This process is enhanced by cytokines, specifically IL8, a potent and specific chemo-tactic stimulus for neutrophils (Caswell *et al.*, 1999). Neutrophils are seen at the site of the cartilage-pannus junction (CPJ), where synovial tissue penetrates the degrading cartilage. Here they contribute to joint destruction (Mohr & Menninger, 1980). Far more neutrophils accumulate in synovial exudates than in surrounding tissue. They produce various cartilage degrading enzymes including MMPs (Choy & Panayi, 2001). Activated neutrophils in the synovial fluid release oxygen free radicals, resulting in damage to the joint via breakdown of hyaluronic acid and inhibition of endogenous protease inhibitors (Weitz *et al.*, 1988).

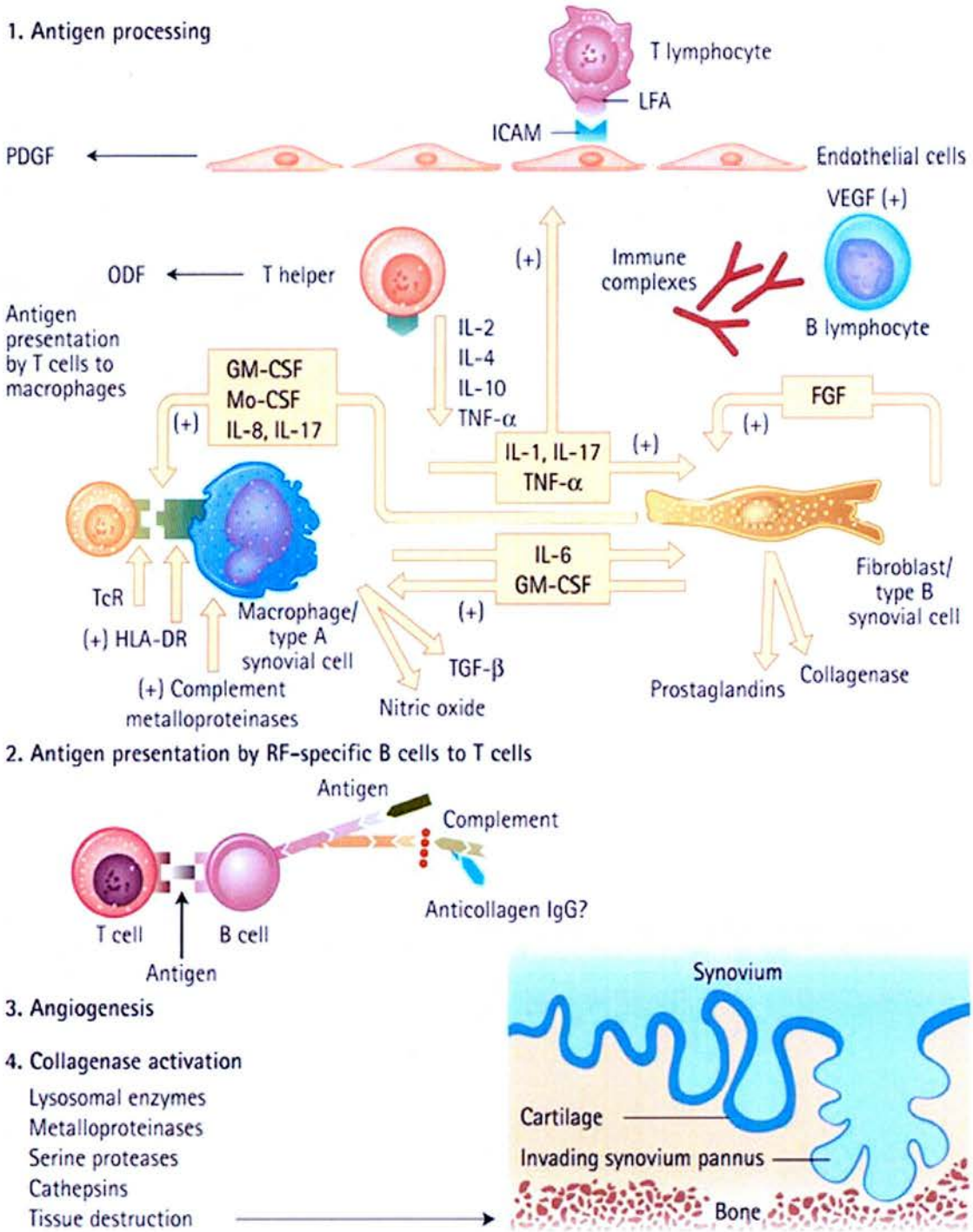


Figure 1.2. The process of synovial inflammation in RA. Exposure of genetically predisposed patients to an unknown trigger initiates a cascade of events leading to an autoimmune response, in which many inflammatory mediators are involved, including interleukins, tumour necrosis factor, fibroblast growth factors, platelet-derived growth factors, monocyte- and neutrophil-stimulating peptides, granulocyte macrophage – colony stimulating factor, PGs and nitric oxide (NO). Illustration from Matteson & Mason, 2005).

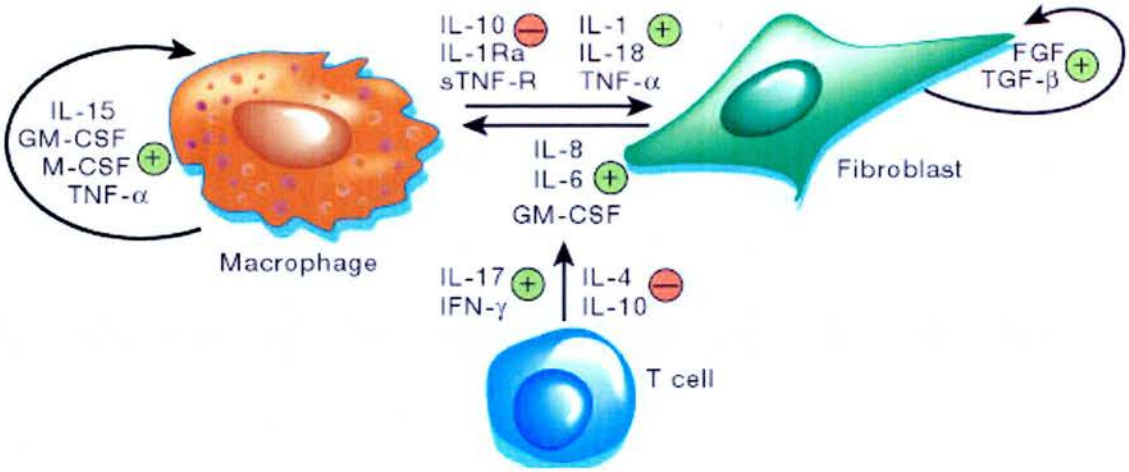


Figure 1.3. Macrophages and fibroblasts are adjacent to one another in the synovial intimal lining and produce cytokines that can activate either themselves or their neighboring cells. Pro-inflammatory cytokines (+) and anti-inflammatory proteins (-) are indicated (Diagram from Firestein, 2003).

B cells, dendritic cells, T cells and macrophages form lymphoid-like structures within the synovial membrane and produce antibodies (Smolen *et al.*, 2005), including rheumatoid factor, an antibody against IgG. These auto-antibodies form immune complexes and are deposited in the cartilage of RA patients (Cooke *et al.*, 1975). The stimulus for maturation of B cells to Ig secreting plasma cells was thought to be CD4+ T cells; however, as stated above, T cells are not activated in the chronic phase of RA. IL6 however is also a potent stimulus for this maturation (Hirano, 1992); thus, IL6 from synovial fibroblasts is likely to be providing the T cell independent stimulus for continuous plasma cell activation and rheumatoid factor production (Bathon, 2005). Plasma cells have been implicated in joint destruction at the CPJ (Kobayashi & Ziff, 1975).

Chondrocytes are activated by IL1 and TNF, resulting in the production of proteolytic enzymes, therefore contributing to the destruction of their own cartilage matrix (Borden *et al.*, 1996). Chondrocyte derived cells are known to appear in the proliferating pannus (Allard *et al.*, 1987). In contrast with enzyme-mediated cartilage degradation, bone destruction depends on osteoclasts (Gravallese, 2002); these cells are not present in the

inflamed joints of non-destructive arthritis. Osteoclasts are formed by fusion with monocytes.

Increased numbers of **mast cells** have been found in the synovial tissue and fluid of patients with RA (Tetlow & Woolley, 1995), especially at the site of cartilage erosion (Bromley *et al.*, 1984). Mast cells are found in connective tissue, and contain numerous basophilic granules and release substances such as heparin and histamine in response to injury or inflammation of bodily tissues. Local accumulations of mast cells were observed in approximately 50% of chronic RA synovial tissue samples from the CPJ (Tetlow & Woolley, 1995); evidence of cell activation was present in most samples. Furthermore, mice that lack mast cells are resistant to inflammatory erosive arthritis induced by arthritogenic serum (Lee *et al.*, 2002). Mast cells contain many inflammatory mediators, which if released could contribute to the rheumatoid lesion. They are the major source of tissue histamine and raised levels of histamine have been recorded both in the peripheral blood and synovial fluid of patients with RA (Partsch *et al.*, 1982).

1.2 CYTOKINES IN RA

Cytokines are small protein molecules produced by many cells that modulate the function of other cell types, see below for more detail and Table 1.1 for a summary. Cytokine production is stimulated by numerous factors, including endotoxin, immune complexes, toxins, physical injury and inflammatory processes such as other cytokines (Billingham, 1987). Cytokines have effects in acute and chronic inflammation. They can act on the same cell that produces them in an *autocrine* fashion; on cells in the immediate vicinity, for example in joint spaces via a *paracrine* effect; or alternatively by an *endocrine* effect, where they travel through the blood and are able to reach all parts of the body, like hormones (Collins, 1999).

Cytokines are produced during immune and inflammatory responses, and secretion of these mediators is transient and closely regulated. They are not stored within cells, but are produced and secreted as necessary. Many cell types make and release multiple cytokines, and each protein is pleiotropic, in that it has a variety of actions on a variety of different cell types. They are multi-functional; each cytokine may have both positive and negative regulatory actions. Cytokines mediate their effects by binding to specific cell-surface receptors on target cells; these receptors can be regulated by both endogenous and exogenous signals. Despite their pleiotropic effects, cytokines can be divided into five groups, based on their major functions, or the nature of the target cell (Collins, 1999):

- *Cytokines that regulate lymphocyte function*: regulate activation, growth and differentiation of lymphocytes, includes IL2, IL4, IL10 and transforming growth factor β (TGFβ).
- *Cytokines involved with natural immunity*: includes TNFα, IL1β, interferon (IFN) α, IFNβ and IL6
- *Cytokines that activate inflammatory cells*: includes IFNγ, TNFα, TNFβ, IL5, IL10 and IL12.
- *Chemokines:* characterized by chemo-tactic activity for leukocytes.
- *Cytokines that stimulate haematopoiesis:* mediate immature leukocyte growth and differentiation. Includes IL3, IL17, granulocyte macrophage – colony stimulating factor (GM-CSF), Monocyte - CSF, granulocyte - CSF and stem cell factor.

The levels of pro-inflammatory cytokines are elevated in the joints and blood of patients with RA (Chikanza *et al.*, 1995a; Eastgate *et al.*, 1988; Houssiau, 1995; Houssiau *et al.*, 1988; Ozaki *et al.*, 2001; Saxne *et al.*, 1988). Anti-inflammatory cytokines also exist, including IL10 and IL4, which cooperate to inhibit the production of pro-inflammatory mediators. Anti-inflammatory cytokines are also elevated in the joints of patients with RA.

In chronic inflammation, such as RA, an imbalance between pro- and anti-inflammatory mediators develops, resulting in cellular damage and destruction of cartilage and bone.

There are many pro- and anti-inflammatory cytokines known to be involved in RA, but for the purpose of this thesis only IL1, IL2, IL4, IL6, IL10, GM-CSF, IFNγ and TNFα will be described, see Table 1.1 and Figures 1.2 and 1.3 for a summary of some key mediators and their roles in RA.

Effects	IL1	TNFα	IL6	GM-CSF	IFNγ
MC/MP activation	↑	↑	_	↑	↑
Mononuclear cell infiltration	↑	↑	↑	↑	↑T-cells only
Synovial vascularization	_	_	↓?	_	
Synovial fibrosis/ hyperplasia	↑	↑	↑?	_	↓
Cartilage breakdown	↑	↑	↑?	↑?	↓
Bone resorption	↑	↑		↑?	↓↑

Key: ↑ = stimulation, ↓=inhibition, ↓↑= conflicting reports, ?=weak effects only observed in vitro - may contribute
MC= monocytes
MP= macrophage.

Table 1.1. Possible roles of some pro-inflammatory cytokines in the RA joint (table modified from Rickard & Gowen, 1993).

1.2.1 TUMOUR NECROSIS FACTOR α (TNFα)

TNFα is produced primarily by monocytes and macrophages, but also by T cells, B cells, Natural Killer cells and fibroblasts (Tracey & Cerami, 1994) and its production can be stimulated by several factors, including lipopolysaccharide (LPS), IL1, GM-CSF, TNFα

itself, hypoxia, oxygen radicals and complement activation. TNFα is a potent cytokine, which exerts a diverse range of effects. It is an autocrine stimulator as well as a potent paracrine inducer of other inflammatory cytokines including IL1, IL6, IL8, GM-CSF and MMPs and collagenase (Brennan *et al.*, 1989; Butler *et al.*, 1995). Furthermore, it induces the production of PGs, increases the expression of adhesion molecules and may facilitate osteoclastogenesis and bone resorption (Dayer *et al.*, 1985; Lader & Flanagan, 1998).

TNFα is found in high levels in RA synovial fluid and pannus (Field *et al.*, 1991) and its expression has been positively correlated with synovitis and erosions (Field *et al.*, 1991; Neidel *et al.*, 1995). Several lines of evidence suggest a crucial role for TNFα in initiating inflammatory arthritis (Feldmann *et al.*, 1996); TNFα transgenic mice, which express human TNFα constitutively, can spontaneously develop an inflammatory, destructive polyarthritis (Keffer *et al.*, 1991). Furthermore, inhibiting TNFα activity constitutively by monoclonal antibodies in these animals prevents the development of spontaneous arthritis in mice (Keffer *et al.*, 1991). In addition, TNFα knockout mice are resistant to the development of experimental synovitis (Henderson & Pettipher, 1989; Hultgren *et al.*, 1998). During collagen-induced arthritis (CIA) in mice, blocking TNFα with a soluble TNF-receptor fusion protein or with monoclonal antibodies ameliorates disease activity and reduces the severity of joint damage (Williams *et al.*, 1992; Wooley *et al.*, 1993b). Moreover, intra-articular injection of TNFα can directly induce joint inflammation and damage in rats (Campagnuolo *et al.*, 2003).

Clinically, anti-TNFα treatment potently inhibits arthritis (Feldmann & Maini, 2003). Currently three TNF antagonists are approved for the treatment of RA: infliximab (a chimeric monoclonal antibody to TNF), adalimumab (a human monoclonal antibody to TNF) and etanercept (a fusion protein of the p75 receptor; for reviews see Alldred, 2001; Emery, 2001; Furst *et al.*, 2003; Goldenberg, 1999; Hochberg *et al.*, 2003; Jobanputra *et al.*, 2002). All three treatments have proved efficacious in patients who failed to respond sufficiently to methotrexate treatment. Infliximab and adalimumab are antibodies to TNFα,

whereas etanercept mimics one of the endogenous TNFα receptors, thus binding free TNFα protein. They work by binding to TNFα and preventing it from interacting with receptors on cell surfaces. This blocks the processes described above, decreasing the overactivity of the immune system (particularly in the joints), helping to relieve joint pain and swelling, and slowing down or even stopping joint damage caused by RA.

1.2.2 INTERLEUKIN 2 (IL2)

IL2 is a T cell-derived cytokine which is present at relatively low levels in the joints of RA patients, compared with levels of macrophage- and fibroblast-derived products (Firestein *et al.*, 1990). Results from IL2 studies in arthritis are conflicting; immunohistochemical analysis of tissue sections from RA patients revealed lymphokine expression in 0.1-0.3% of T cells, particularly IL2 and IFN γ (Steiner *et al.*, 1999). Furthermore, considerable IL2-like activity is detected in the synovial fluid but not in the peripheral blood (Boyum, 1968; Lefkovits & Waldmann, 1979; Wood *et al.*, 1983). *In vitro* production of IL2 in cultures of synovial fluid lymphocytes has been reported as diminished (Bolhuis *et al.*, 1978) and enhanced (McKenna *et al.*, 1986; Wood *et al.*, 1983). Furthermore, IL2-responsiveness of synovial fluid lymphocytes has been shown to be increased by some groups (Gillis *et al.*, 1978), but reduced by others (Taetle & Royston, 1980). Adjuvant-induced arthritis in rats, a T-cell dependent disease, leads to T-cell activation and proliferation, processes in which the *de novo* expression of the IL2 receptor plays a pivotal role. Intra-peritoneal EL2-PE40, a cytotoxic IL2-Pseudomonas endotoxin fusion protein, proved to be an effective and specific modifier of adjuvant arthritis, based on clinical, histological and radiographic assessment (Case *et al.*, 1989).

1.2.3 INTERLEUKIN 4 (IL4)

IL4 is an anti-inflammatory cytokine produced by CD4+ T cells and participates in the differentiation and growth of B cells (Isomaki & Punnonen, 1997). *In vitro* IL4 inhibits the activation of T cells which, in turn, decreases the production of IL1 and TNFα and inhibits cartilage damage (van Roon *et al.*, 1996). Furthermore, IL4 inhibits the production and functions of IL6 and IL8 (Ralph *et al.*, 1992; Sugiyama *et al.*, 1995; Vannier *et al.*, 1992), induces T helper cells and inhibits MMP production. In cultures of synovium samples from patients with RA, IL4 increased the expression of IL1 receptor antagonist (Vannier *et al.*, 1992) and soluble TNFα receptors (Joyce *et al.*, 1994) both of which reduce inflammation (Chomarat *et al.*, 1995). Recombinant IL4 has been tested in patients with RA; however, the clinical efficacy of the treatment has been disappointing, perhaps due to the short half life of the substances (Choy & Panayi, 2001).

1.2.4 INTERLEUKIN 10 (IL10)

IL10 is another anti-inflammatory cytokine which is produced by monocytes, macrophages, B cells and T cells. It inhibits the production of several other cytokines, including IL1 and TNFα and the proliferation of T cells *in vitro* (Isomaki & Punnonen, 1997). IL10 can also reverse the cartilage degradation mediated by antigen-stimulated mononuclear cells from patients with RA (van Roon *et al.*, 1996). Although IL10 is found in the synovial fluid of these patients (Cush *et al.*, 1995; Isomaki *et al.*, 1996), the amount may be insufficient to suppress inflammation (Katsikis *et al.*, 1994). Like IL4, recombinant IL10 has been tested in patients with RA, in a phase I dose-ranging study in patients with active RA, administration of recombinant human IL-10 at a dose of 5 $\mu g\ kg^{-1}$ by daily subcutaneous injection resulted in a trend towards improvement in disease activity, compared with placebo recipients, after 4 weeks of administration (Maini *et al.*, 1997).

1.2.5 GRANULOCYTE MACROPHAGE – COLONY STIMULATING FACTOR (GM-CSF)

GM-CSF is a growth factor synthesized by macrophages, fibroblasts, endothelial cells and activated lymphocytes (Groopman *et al.*, 1989). As well as promoting the growth and differentiation of granulocytes and macrophages, it is known to have several other pro-inflammatory roles, including increasing the secretion of IL1, TNFα and prostaglandin E_2 (PGE_2) release from macrophages (Fischer *et al.*, 1988; Heidenreich *et al.*, 1989) as well as amplification of cartilage destruction by IL1 and TNF (Alvaro-Gracia *et al.*, 1989).

1.2.6 INTERFERON γ (IFNγ)

It is likely that IFNγ, as a T cell-derived cytokine, is involved in the early stages of RA. IFNγ specifically inhibits TNFα mediated matrix degradation therefore lack of IFNγ and over-production of TNFα by tissue macrophages suggests that the destructive actions of TNFα proceed almost unopposed. In bone and cartilage IFNγ inhibits matrix resorption by blocking osteoclast precursor proliferation and multinucleated osteoclast formation and by reducing MMP synthesis respectively. IFNγ appears to have no more therapeutic value than placebo in the management of RA, because its effectiveness is dependent upon disease stage and its ability to block only the actions of TNFα and not those of IL1 (Veys *et al.*, 1997).

1.3 INTERLEUKIN 1 (IL1)

1.3.1 FORMATION

There are two distinct related IL1 glycoproteins, IL1α and IL1β, which are encoded by separate genes; in most human tissues IL1β messenger ribonucleic acid (mRNA) predominates (Arai *et al.*, 1990). The gene which encodes IL1β is not spontaneously expressed but can be stimulated by LPS, complement components or IL1β itself. IL1α and IL1β are synthesized as 31 kilo Dalton (kD) precursors (pro-IL1) and converted to 17 kD

active glycoproteins, by interleukin converting enzyme (ICE), also known as caspase-1, which is present in many cell types. Pro-IL1α can be found on the surface of cells in a biologically active form, whereas pro-IL1β requires cleavage for its optimal secretion and biological activity (Dinarello, 1994a). IL1 can be produced and released by a range of cells including macrophages, fibroblasts, keratinocytes, T- and B-lymphocytes, astrocytes, microglia, endothelial cells, smooth muscle cells and chondrocytes.

1.3.2 RECEPTORS

There are two main receptors of the IL1 family, IL1 receptor type I and type II (IL1 RI and IL1 RII respectively), they are encoded by a common receptor gene (Sims *et al.*, 1995). Both receptors have a single transmembrane domain, and the extracellular portions of each receptor are homologous (26-28%) and contain three Ig-like domains. However, whereas the IL1 RI has a long cytosolic domain (213 amino acids; Sims *et al.*, 1988) that of the IL1 RII is relatively short (29 amino acids; McMahan *et al.*, 1991). It is therefore not surprising that IL1 RII is not involved in cell-signalling pathways (Sims *et al.*, 1993).

IL1 RI is an 8 kD glycoprotein found at low levels on nearly all cells. It is heavily glycosylated and blocking the glycosylation sites reduces the binding of IL1 (Mancilla *et al.*, 1992). Primary cells usually express less than 200 and in some cases less than 50 receptors per cell; IL1 signal transduction has been observed in cells expressing less than 10 per cell (Dinarello, 1994b). However, the number of cell surface receptors rises rapidly on exposure to inflammatory mediators including IFNγ, IL1, IL2, IL4, PGE_2, platelet derived growth factor (PDGF), vitamin D_3, phorbol esters and promoters of inflammation e.g. LPS (Akahoshi *et al.*, 1988; Koch *et al.*, 1992a). In some cells IL1 down-regulates the number of its own surface receptors by a decrease in mRNA half life (Akahoshi *et al.*, 1988; Koch *et al.*, 1992b; Ye *et al.*, 1992).

IL1 RII is found primarily on the surface of neutrophils, monocytes and B lymphocytes (McMahan *et al.*, 1991) and like the IL1 RI, the number of receptors is increased during inflammation by several mediators including IL1, PGE_2 and IL4 (Re *et al.*, 1994). IL1 RII can be shed from the cell surface and is often found as a soluble receptor in the circulation, here is acts as a "decoy" receptor; it has a much higher affinity for IL1 than IL1 RI and such binding is irreversible, thus is thought to be a regulatory mechanisms for excess IL1 in inflammatory conditions (Colotta *et al.*, 1993). However, soluble forms of both receptors have been detected in human inflammatory fluids and experimental cell culture supernatants (Colotta *et al.*, 1996; Symons *et al.*, 1991; Symons *et al.*, 1990).

1.3.3 SIGNAL TRANSDUCTION

There are less than 100 cell surface IL1 receptors on most cells, and less than 5% of these need to be occupied for a biological response, therefore IL1 signal transduction is highly efficient and greatly amplified (Dinarello, 1994a). The most likely mechanisms for signal amplification is multiple and sequential phosphorylations or dephosphoylations of kinases, which result in nuclear translocation of transcription factors and activation of proteins participating in translation of mRNA.

Within a few minutes of binding to cells, IL1 induces several biochemical events, responses include hydrolysis of guanyltriphosphate-binding protein with no associated increase in adenyl cyclase (AC; O'Neill *et al.*, 1990), activation of AC (Chedid & Mizel, 1990; Mizel, 1990), hydrolysis of three phospholipids by non-phosphatidylinositol phospholipase C (PLC; Kester *et al.*, 1989; Rosoff *et al.*, 1988) and release of arachidonic acid from phospholipids via cytosolic phospholipase A_2 (PLA_2) activation (Gronich *et al.*, 1994).

IL1 does not stimulate hydrolysis of phosphatidylinositol or an increase in intracellular calcium. Some IL1 signalling events are prominent in different cells, post-receptor signalling mechanisms may therefore provide cellular specificity.

1.3.4 REGULATION

Modulation or inhibition of the effects of IL1 can be achieved by reducing the amount of IL1β released, down-regulation of IL1 receptors, antagonism of IL1 at a receptor level, modulation of its actions by anti-inflammatory cytokines, or inhibition of ICE to decrease the amount of active IL1β released from its inactive pre-cursor form.

IL1 Receptor Antagonist

An endogenously released inhibitor of IL1 activity was detected in the serum of humans injected with LPS (Andersson *et al.*, 1992) and in the urine of patients with monocytic leukaemia (Seckinger *et al.*, 1987), this is the IL1 receptor antagonist (IL1Ra). It is a 17 kD protein which is more closely related to IL1β (26% homology) than IL1α (18% homology), and can bind the same receptors (Evans & Robbins, 1994; Graves *et al.*, 1990; Priestle *et al.*, 1989; Vigers *et al.*, 1994). However, IL1 Ra cannot initiate an intracellular signalling cascade (Evans & Robbins, 1994), thus it competes with IL1β for its receptor and acts as a competitive antagonist.

IL1Ra reduces the severity of a number of inflammatory diseases including adjuvant-induced arthritis in rats (Schwab *et al.*, 1991). Levels of IL1Ra increase in inflammatory diseases in animals and humans (Fischer *et al.*, 1992) and therefore may act to regulate the biological effects of IL1. However, the levels are deficient relative to total production of IL1 (Bresnihan & Cunnane, 1998; Chikanza *et al.*, 1995b; Firestein *et al.*, 1994), therefore the administration of exogenous recombinant IL1Ra restores the balance between IL1 and IL1Ra and is a therapeutic approach that has been investigated in recent

years. Natural and recombinant IL1 Ra were shown to inhibit IL1 mediated bone resorption and PGE_2 production (Seckinger *et al.*, 1990). Anakinra has been approved for the treatment of RA (for review see Furst, 2004); it improved clinical symptoms with a 10% reduction in the number of swollen joints in patients as well as slowing the progression of the disease (Bresnihan *et al.*, 1998). However, the lack of an impressive clinical response, despite reductions in disease progression, may indicate that there is a difference between cytokines involved in pain and inflammation and cytokines involved in joint destruction. Immunohistological studies indicate IL1 is present in 90% of cells at the CPJ, the majority macrophages. In addition, 65% of chondrocytes in the superficial layer of the articular cartilage adjacent to the pannus also contain IL1. Similar studies reveal the presence of IL1 Ra in less than 10% of the cells in the above locations, yet a 50% inhibition of IL1 activity requires approximately 100-fold excess of IL1 Ra (Arend *et al.*, 1990; Seckinger *et al.*, 1990).

Soluble IL1 receptor accessory protein

A human soluble IL1 receptor accessory protein (IL1 RAcP) has been described that is recruited to IL1 RI (Greenfeder *et al.*, 1995). The formation of a complex including IL1, IL1 RI and IL1 RAcP appears to be essential for the initiation of IL1 signal transduction (Martin & Falk, 1997). This is supported by evidence that a cell line containing a functional IL1 R1 but not expressing complete IL1 RAcP is unable to respond to IL1 stimulation (Korherr *et al.*, 1997; Wesche *et al.*, 1997). The inhibition or reduction of this molecule could therefore modulate IL1 responses, as shown by Smeets *et al.* (2005). The latter paper demonstrates that he sIL1RAcP ameliorates experimental arthritis without affecting T cell immunity, which is in contrast to IL1Ra.

Anti-inflammatory cytokines

IL4 was found to down regulate the LPS-evoked production of IL1 by human peripheral monocytes in a dose-dependent manner (Essner *et al.*, 1989; te Velde *et al.*, 1990) as well as up regulating the production of IL1Ra protein four-fold and the expression of IL1Ra mRNA two-fold in the same *in vitro* preparation as above (Vannier *et al.*, 1992). In addition, studies using monoclonal antibodies have demonstrated that IL-4 antagonized the action of IL-1 by inducing expression and release of IL-1R II (Colotta *et al.*, 1993), thus reducing the effects of IL1.

IL10, IL 13 and TGFβ act in similar ways to IL4, down regulating IL1 production and up regulating IL1 Ra production (Burd *et al.*, 1995; Colotta *et al.*, 1996; de Waal Malefyt *et al.*, 1991; Girard *et al.*, 1996; Jenkins *et al.*, 1994; Vannier *et al.*, 1996).

Inhibition of Interleukin converting enzyme

Inhibition of ICE would result in a reduction in the amount of biologically active IL1 produced. The tetrapeptide ICE inhibitor YVAD blocked the increased expression of cyclooxygenase (COX) 2 and the production PGE_2 in the central nervous system (CNS) of rats following peripheral inflammation or intrathecal injection of IL1 (Samad *et al.*, 2001). In addition, the irreversible tetrapeptide ICE inhibitor, YVAD-CMK, strongly reduced IL1 and IL18 production in human OA cartilage explants (Saha *et al.*, 1999). However this compound is not effective when administered orally. In contrast, pralnacasan (HMR 3480/VX-740) is an orally bioavailable pro-drug of RU 36384/VRT-18858 which inhibits LPS-induced IL1 release by human peripheral blood mononuclear cells *in vitro* (Loher *et al.*, 2004; Rudolphi *et al.*, 2003). *In vivo*, oral administration of pralnacasan in mice inhibited LPS-evoked elevation of serum IL1 by up to 80%. In CIA, prophylactic treatment with pralnacasan delayed the onset of forepaw inflammation, and reduced disease severity by 50–70%. Furthermore, pralnacasan significantly reduced forepaw inflammation and progression of arthritis when administered to mice with established arthritis reducing the incidence of

cartilage damage by >60% and bone erosion by >80%, compared with vehicle-treated mice (Ku *et al.*, 2001). Moreover, pralnacasan treatment significantly ameliorated histopathological damage of the knee joint in two mouse models of OA (CIA and spontaneous OA in STR/1N mice; Rudolphi *et al.*, 2003).

RU 36384/VRT-18858 is the first potent, selective, non-peptide inhibitor of ICE in phase II clinical trials for RA (Wollheim, 2001). An initial Phase I/IIa clinical program has shown that pralnacasan is well tolerated in healthy volunteers and patients with RA. However, the trial was suspended after animal toxicology suggested likely pralnacasan-associated liver abnormalities after nine-month exposure to high doses of the drug.

Decoy receptors

Development of recombinant human IL1 RI has provided a possible drug treatment for RA, it has been tested in phase 1 trials of patients with active RA, however the efficacy of this agent has not proved promising (Drevlow *et al.*, 1996). The lack of efficacy of recombinant human IL1 RI might be the result of complex interactions occurring within the IL1 signal transduction system. In addition to binding IL1, soluble receptors also bind IL1 Ra; this interaction might complicate the therapeutic use of soluble IL1 receptors, particularly type 1 receptors, as its affinity for IL1 Ra is greater than for IL1β or IL1α (Arend *et al.*, 1994).

1.3.5 ROLE OF IL1 IN INFLAMMATION AND RA

IL1β plays an important role in the pathophysiology of RA by contributing to inflammation as well as joint destruction. Most clinical and pre-clinical evidence relating to the role of IL1 in inflammation and RA relates to IL1β. Infusion of IL1β induces and exacerbates arthritis in animal models (Hom *et al.*, 1988; Hom *et al.*, 1990; Schwab *et al.*, 1991). However, continuous infusion of IL1α in rabbit knees for two weeks also induced

arthritis with changes similar to those seen in RA (Feige *et al.*, 1989) indicating a separate effect of IL1α.

Elevated plasma levels of IL1β have been demonstrated to positively correlate with measures of disease activity (Eastgate *et al.*, 1988) and radiographic progression over one year (Hopkins *et al.*, 1988). Concentrations of IL1β are higher in joint tissue biopsies from patients with joint erosions than those with non-erosive disease (Fong *et al.*, 1994) but at present the measurement of IL1α in plasma offers little clinical value.

IL1 is a key mediator of synovial inflammation. It is responsible for:

- Triggering the recruitment of PMNs, lymphocytes and monocytes into the joint
- The activation of macrophages, resulting in release of proteases (Bresnihan & Cunnane, 1998)
- stimulating T- and B-cell proliferation and differentiation (Dinarello, 2002)
- Enhancing the proliferation of fibroblasts, leading to pannus formation.

IL1 plays a key role in cartilage and bone destruction during RA. A primary action of IL1 is the stimulation of MMP synthesis by articular chondrocytes, synovial macrophages, fibroblasts, invading macrophages and neutrophils, which causes proteoglycan degradation, which in turn drives cartilage destruction (Gurr *et al.*, 1988). Furthermore reactive oxygen species are released which are efficient factors in cartilage matrix destruction as previously described. A further effect of IL1 on chondrocytes is to inhibit proteoglycan synthesis and the production of MMP inhibitors preventing the normal maintenance of cartilage (Bresnihan *et al.*, 1998; Eastgate *et al.*, 1988; van de Loo *et al.*, 1995; van de Loo *et al.*, 1992; Van Lent *et al.*, 1995). In experimental models, this loss of cartilage has been shown to occur early in the disease progress (van den Berg, 2001). In addition, erosion of the cartilage surface is mediated by the formation of pannus and increased migration of PMNs in to the synovial tissue; IL1 has an important role in driving these processes. In addition, IL1 is a potent

stimulator of synoviocytes, chondrocytes and osteoblasts, cells responsible for bone formation.

The effects of IL1 on bone resorption are mediated indirectly through interaction of osteoprotegrin (OPG) and osteoprotegrin ligand (OPG-L) and their effects on osteoclasts. OPG stimulates induction of osteoclast differentiation and activation. When bound to OPG-L bone resorption is inhibited, thus OPG-L/OPG balance determines the relative degree of erosion. IL1 acts directly on T cells and osteoclasts to increase expression of OPG-L, which then stimulates the differentiation of osteoclast precursors to mature osteoclasts; furthermore, it increases their resorptive activity (Gravallese & Goldring, 2000; Kong *et al.*, 1999). IL1 has been shown to induce osteoblast apoptosis and may thereby prevent new bone formation, which is particularly important when repair is required (Lemonnier *et al.*, 2001; Tsuboi *et al.*, 1999).

IL1 initiates the production of COX-2, PLA_2 and inducible nitric oxide synthase (iNOS). This accounts for the large amount of PGE_2, platelet activating factor (PAF) and nitric oxide (NO) produced by cells exposed to IL1 or in animals or humans injected with IL1. Furthermore, *de novo* synthesis of COX-2 was enhanced by IL1β in rheumatoid synovial explant cultures and cultured rheumatoid synoviocytes (Crofford *et al.*, 1994). The PG products of COX-2 contribute to pain and inflammation and high levels of NO kill chondrocytes, the cells responsible for cartilage remodelling (Notoya *et al.*, 2000). COX-2 is up regulated in rats with adjuvant-induced arthritis (Sano *et al.*, 1992) and in the synovium of patients with RA (Crofford *et al.*, 1994).

Intra-articular injection of IL1 in rabbits induces a transient infiltration of neutrophils into the joint space, followed by mononuclear cell infiltration (Pettipher *et al.*, 1986). Loss of proteoglycans from the articular cartilage also follows IL1 injection into the joint (Pettipher *et al.*, 1986). In addition, it has been shown that CIA in mice is accelerated by subcutaneous injection of IL1 (Hom *et al.*, 1988).

In contrast, it has also been observed that injection of IL1 into the knee joints of rats with adjuvant-induced arthritis leads to a reduction in inflammation and joint destruction (Jacobs *et al.*, 1988). This effect was observed with both pre-treatment and administration of IL1 once the arthritis was present. A similar study was carried out in Streptococcal Cell Wall (SCW) arthritis, and demonstrated anti-inflammatory effects of IL1 (Schwab *et al.*, 1991). Furthermore, it has been demonstrated that intra-venous or subcutaneous IL1 inhibits carageenan-induced paw oedema in a dose-dependent manner (Drelon *et al.*, 1994; Drelon *et al.*, 1992; Nakamura *et al.*, 1988). In Nakamura's studies oedema inhibition is concominant with the increase of plasma levels of adrenocorticotophic hormone and corticosterone, and hence anti-inflammatory actions of IL1 may be, at least in part, explained by pituitary-adrenal axis simulation.

Numerous experimental studies provide evidence that proinflammatory cytokines induce or facilitate inflammatory as well as neuropathic pain or hyperalgesia. Direct receptor-mediated actions of cytokines on afferent nerve fibres have been reported as well as cytokine effects involving further mediators. For example, electrophysiological recording from rat DRGs during topical application of IL1β resulted in an increase in the discharge rate, increased mechanosensitivity of the DRG units (Ozaktay, 2006). Furthermore, brief applications of IL1β to nociceptive neurons yielded a potentiation of heat-activated inwad currents and a shift of activation thresholds towards lower temperatures without altering intracellular calcium levels, the effect was not mediated by G-protein coupled receptors but was mediated by activation of protein kinases (Obreja *et al.*, 2002; for review of cytokines and pain see Sommer & Kress, 2004).

1.4 INTERLEUKIN 6

1.4.1 FORMATION

IL6 is produced from a single gene encoding a product of 212 amino acids, which is cleaved at the N-terminus to produce a 184 amino acid peptide with a molecular weight of between 23 and 32 kD (Yasukawa *et al.*, 1987). Extensive posttranslational modifications occur and may account for the variability of the end product.

IL6 production has been documented from many cells, including fibroblasts (Weissenbach *et al.*, 1980), endothelial cells (Corbel & Melchers, 1984), keratinocytes (Baumann *et al.*, 1984), monocytes/macrophages (Aarden *et al.*, 1987; Baumann *et al.*, 1984), lymphocytes (Hirano *et al.*, 1985), mast cells (Plaut *et al.*, 1989), synoviocytes (Guerne *et al.*, 1989) a variety of tumour cell lines (Hirano *et al.*, 1986), and many more. However, synoviocytes, T cells and B lymphocytes are the primary sources of IL6 within the synovium (Guerne *et al.*, 1989; Hirano *et al.*, 1988).

1.4.2 RECEPTORS

Specific binding of IL6 has been demonstrated in a range of cells. However, IL6 also binds soluble receptors (sIL6R). Normal (non-tumour) cells express only a few hundred IL6 receptors each (Van Snick, 1990); IL6 Rs have been found on T and B lymphocytes as well as macrophages. Interestingly, the expression of IL6Rs is regulated differentially in B and T lymphocytes. T cells down regulate IL6Rs upon activation, whereas B cells acquire them only at the final stages of maturation (Zhang *et al.*, 1988), indicating that IL6 acts early in T cell activation and late in B cell responses.

The sIL6R is a 55 kD protein generated from differential RNA splicing or proteolytic cleavage and shedding of cell surface bound receptors (Jones *et al.*, 2001; Mullberg *et al.*, 1993). Production of sIL6Rs is increased in RA as well as other

inflammatory conditions (De Benedetti *et al.*, 1994; Pignatti *et al.*, 2003). Synovial fluid levels of sIL6R are elevated in RA compared with OA (De Benedetti *et al.*, 1994).

1.4.3 SIGNAL TRANSDUCTION

The cytokine-cytokine receptor complex binds to a cell surface glycoprotein (gp), gp130 (Kishimoto *et al.*, 1995). Cellular activation results from the activation of gp130; the intracellular domain of the 80 kD receptor plays no role in the transduction of the IL6 signal (Taga *et al.*, 1989). Following binding of IL6 to the receptor, and subsequent association of gp130, the Janus kinase (JAK) family protein tyrosine kinase(s) are activated and tyrosines are phosphorylated on various cellular proteins including gp130 itself. The activated tyrosine kinases, in turn, phosphorylate and activate the signal transducer and activator of transcription family proteins, after a complex series of intercellular events the downstream effects of IL6 are seen (Inoue *et al.*, 1997).

1.4.4 REGULATION

IL6 is not produced constitutively by normal cells, but its expression is rapidly induced by viral infections (Cayphas *et al.*, 1987; Frei *et al.*, 1989; Sehgal *et al.*, 1988), LPS (Nordan & Potter, 1986), and a variety of other stimuli including pro-inflammatory cytokines such as IL1 α and β (Shalaby *et al.*, 1989), TNFα (Van Damme *et al.*, 1987), IFNγ (Sanceau *et al.*, 1989; Shalaby *et al.*, 1989), PDGF (Kohase *et al.*, 1987), IL3 and GM-CSF (Van Snick, 1990). However, not all cells respond in the same way to all these factors, for example IL1 is the most potent inducer of IL6 in fibroblasts but induces very little IL6 in bone marrow cells, which respond highly to IL3 or GM-CSF.

The different effects of IL6 may be partly mediated by the level of sIL6R, it could be the level of this receptor, rather than IL6 itself, that determines the biological response.

1.4.5 ROLE OF IL6 IN INFLAMMATION AND RA

Despite high levels of IL6 production in joints and serum from patients with RA, in animals with experimental arthritis, and the correlation with disease activity or radiological joint damage (Hirano *et al.*, 1988; Houssiau *et al.*, 1988; Swaak *et al.*, 1988), the role of IL6 during inflammation is largely unclear. Levels of IL6 decrease after effective treatment with DMARDs (Dasgupta *et al.*, 1992; Kotake *et al.*, 1996). However, the precise pathogenic role of IL6 is controversial because it has both pro- and anti-inflammatory properties *in vitro.*

IL6 acts synergistically with IL1 and TNFα to augment the production and release of MMPs and increase the production of tissue inhibitor of MMP from human synovial fibroblasts (Ito *et al.*, 1992). Therefore IL6 may modulate the balance between MMPs and inhibitors of MMPs at sites of inflammation in RA; it is this imbalance that may result in joint and cartilage destruction.

IL6 is produced by osteoblasts and chondrocytes and is induced by pro-inflammatory cytokines (Guerne *et al.*, 1990; Hierl *et al.*, 1998; Littlewood *et al.*, 1991). In bone, IL6 is an autocrine and paracrine factor that plays a role in osteoclast-mediated bone resorption (Ishimi *et al.*, 1990; Roodman *et al.*, 1992). IL6 does not appear to have a direct effect in stimulating bone resorption mediated by mature osteoclasts (al-Humidan *et al.*, 1991; Bertolini *et al.*, 1994). In cartilage, IL6 along with other pro-inflammatory cytokines has been shown to be significantly up regulated in OA *versus* normal joints (Moos *et al.*, 1999). Synovial fluid with high levels of IL6 promotes osteoclast activation and was shown to promote osteoclast cell formation when added to co-cultures of osteoblasts and bone marrow cells (Kotake *et al.*, 1996). The degree of activation correlates with joint damage in these patients (Kotake *et al.*, 1996).

IL6 is a potent B-cell growth and differentiation factor. Within the joint, IL6 stimulates the differentiation of B cells to plasma cells (Hirano *et al.*, 1986), which may

account for the high levels of rheumatoid factor. In addition, IL6 stimulates proliferation and differentiation of T lymphocytes into cytotoxic T cells (Okada *et al.*, 1988).

Another pro-inflammatory action of IL6 is the stimulation of hepatocytes to produce acute phase proteins (APP), which is generally measured by the concentration of C-reactive protein (CRP). IL6 is the only cytokine responsible for triggering APP release (Castell *et al.*, 1988), mediating the innate immune response. Furthermore, IL6 promotes leukocyte chemotaxis (Taga & Kishimoto, 1997), by inducing the expression of intercellular adhesion molecule-1 (ICAM-1) and other adhesive ligands.

IL6 knockout mice are protected against joint inflammation and destruction in both collagen- and antigen-induced models of arthritis (Alonzi *et al.*, 1998; de Hooge *et al.*, 2000; Ohshima *et al.*, 1998). This protection was seen despite the expression of both TNFα and IL1 in the inflamed synovium (Ohshima *et al.*, 1998), demonstrating the importance of IL6 in these models of disease. Furthermore, an IL6 receptor neutralizing antibody suppressed the onset and reduced the severity of CIA in mice (Takagi *et al.*, 1998).

The above observations suggest that inhibition of the activity of IL6 in patients with RA may be of clinical benefit. IL6 blockade using a monoclonal antibody was well-tolerated and resulted in significant improvement in clinical scores and laboratory parameters in a small number of patients with RA (Wendling *et al.*, 1993). A recent therapeutic trial of an anti-IL6 receptor monoclonal antibody in patients with active RA resulted in a significant, dose-dependent reduction in disease activity (Choy *et al.*, 2002).

However, there is also evidence that IL6 has anti-inflammatory effects. *In vitro* IL6 can reduce inflammation by suppressing IL1 and TNFα production and by inducing the release of IL1 Ra and soluble TNFα receptors (Taga & Kishimoto, 1997). Hence it has been argued that IL6 may regulate rather than mediate inflammation. In addition in zymosan-induced arthritis, IL6 knockout mice had increased cartilage degradation, which occurred in spite of reduced joint swelling and normal production of IL1, TNF and NO (van de Loo *et*

al., 1997). IL6 may therefore have both deleterious and protective effects in inflammatory arthritis.

Limited research on arthritis can be conducted in patients due to ethical constraints. Most research is carried out in laboratory animals; hence, there have been several experimental models of joint inflammation developed to mimic human joint diseases. The next section will review some commonly used models of inflammatory arthritis, their advantages and disadvantages as well as the similarities to human conditions.

1.5 EXPERIMENTAL ANIMAL MODELS OF RA

Rodent models of RA serve as valuable tools to investigate the underlying mechanisms at early, intermediate and late stages of joint disease (for review see Sakaguchi and Sakaguchi, 2005 and Wooley, 2004). Several animal models of joint inflammation have been developed and characterised, although none of these mimic the exact aetiology of RA in humans, which probably involves several different initiating factors. However, they can provide a valuable insight to help unravel the mechanisms of pain and inflammation as well as the progression of the disease itself. Most experimental RA in animals is produced with an inducing agent, however, the advancements in genetics mean genetically altered mice can be engineered which spontaneously develop RA-like disease.

1.5.1 ADJUVANT-INDUCED ARTHRITIS

Jules Freund (1947) introduced a mixture of mineral oils, heat killed mycobacterium and emulsifying agents, known as Freunds complete adjuvant (FCA: Freund, 1956; Freund, 1947). This concoction proved to be an efficient enhancer of cell-mediated and humoral immune responses towards antigens with which it was emulsified. The first model of polyarthritis was developed by Storerk *et al* (1954) who reported that joint lesions developed

in rats after immunization with FCA and rat spleen tissue. He suspected that the spleen tissue was arthritogenic, but Pearson and Wood (1959) found that injection of rats intra-dermally at the tail base with complete adjuvant alone induced a chronic, lapsing-remitting arthritis involving multiple joints, this is the poly-arthritis adjuvant arthritis model (Pearson, 1956; Stoerk *et al.*, 1954). Although this initially manifested as a profound inflammation at the site of injection, it also resulted in a delayed T-cell mediated hypersensitivity reaction (Pearson & Wood, 1959; Wall, 1984) with multiple joint involvement and subsequent development of lesions of the eyes, ears, nose, skin, genitals, spleen, liver and bone marrow as well as lymph node enlargement and severe weight loss. Adjuvant-induced polyarthritis has many disadvantages, particularly the widespread systemic disease which causes severe discomfort and stress to the animal (Butler *et al.*, 1992; Stein *et al.*, 1988).

The model was subsequently refined to a monoarthritic model in the ankle joint of rats, removing some of the complications of the model. Most refined models use local injection of FCA into (Butler *et al.*, 1992) or around the tibiotarsal joint (Donaldson *et al.*, 1993; Grubb *et al.*, 1991). In addition, it was noted that increasing doses of FCA resulted in symmetry of joint inflammation across the body following direct injection to the rat ankle joint (Donaldson *et al.*, 1993), which was more humane than polyarthritis, yet mimicked human RA more closely. Subsequently the unilateral model was extended to the knee joint, which is now a well characterized model of experimental arthritis in rats and mice (Gauldie *et al.*, 2004; Wilson *et al.*, 2006).

Heat shock proteins have been implicated in the pathogenesis of arthritis in humans and rodents. The 65 kD mycobacterial heat shock protein in FCA may be the primary autoantigen in this experimental model (van Eden *et al.*, 1988). Furthermore, it has been shown that a specific epitope of mycobacterial heat shock protein 65 corresponding to certain amino acids is recognised by rat T cells (van Eden *et al.*, 1988).

FCA-induced arthritis shares many features with human RA including genetic linkage, synovial CD4+ T cells and T cell dependence (see Table 1.2; Goodson *et al.*, 2003).

However, one of the major differences between the adjuvant arthritis model and human RA is simply that the inciting agent is known in the model.

It is the adjuvant-induced arthritis model that will be used throughout this thesis, as it has been the most extensively studied and characterized in this laboratory in the past, therefore experimental work in this thesis can be correlated with and supplemented by previous research, with the aim of reducing the number of animals needed for the studies, in line with Home Office requirements and the 3 R's.

			Adjuvant arthritis	**Rheumatoid Arthritis**
Clinical	Acute and recurrent arthritis	Peripheral joints	++	++
		Spinal joints	+	++
	Chronic deforming arthritis		++	++
	Skin lesions		+	0
	Genital lesions		++	0
	Eye lesions		+	+
	Progressive and destructive joint disease		++	++
	Skin and s.c. nodules	Rheumatoid nodules	++	++
		Vasculitis only	+	0
Pathologic	Acute and sub-acute synovitis		++	++
	Immune mediated	Primarily mononuclear cell response	++	++
		T-cell dependent	++	++
		B-cell dependent	++	++
	Invasion of bone and joint space by pannus		++	++
	Bursitis and tendonitis		++	++
	Osteitis and periostitis ankylosis	Fibrous	++	+
		Bony	+	+
	Changes in serum proteins		++	++
	Rheumatoid factor		0	++
0 =almost never, + = rarely, ++ = common				

Table 1.2 The clinical and general pathological features of RA and poly-adjuvant-induced arthritis. The model used during this thesis was monoarthritis, which does not include the widespread, systemic features noted in this table. Examination of the table reveals the only real difference between adjuvant arthritis and RA are the absence of rheumatoid factor in adjuvant-arthritis and the lack of genital or skin lesions in RA. Despite these differences an overwhelming number of features occur in both diseases (modified from Pearson (1966).

1.5.2 RAT STREPTOCOCCAL CELL WALL (SCW) ARTHRITIS

Schwab and colleagues described SCW arthritis in the 1970s and subsequently Schwab and Wilder separately characterized important features of this model (Schwab,

1995; Wilder, 1988). Intra-peritoneal injection of SCW mix induces a chronic, severe erosive arthritis in female Lewis rats. However, male Lewis rats are less susceptible, and there are vast differences between strains of rats (Wilder, 1988), so although it is a good model for human RA, there are limitations on animal strain, but the unknown genetic mechanisms for this variation may help elucidate human susceptibility to RA.

1.5.3 COLLAGEN INDUCED ARTHRITIS (CIA)

CIA is a model of RA that is induced in susceptible mouse strains following intradermal immunization with collagen-II emulsified in an adjuvant; it is an extensively studied animal model of RA because it shares both immunological and pathological features of human RA (Stuart *et al.*, 1985). Following immunization with collagen type II animals develop an erosive, polyarticular arthritis mediated by an autoimmune response (Courtenay *et al.*, 1980). The significance of this model is that collagen type II is the major constituent protein of cartilage at sites of inflammation in RA. Unlike the models of adjuvant- and antigen-induced arthritis which are largely, if not entirely, mediated by T cells, CIA permits the study of synovitis induced by antibodies and propagated by T cells specific to a major glycoprotein found only in cartilage (Myers *et al.*, 1997).

1.5.4 MOUSE TRANSGENIC AND KNOCKOUT MODELS OF RA

Deletion or introduction of genes for particular receptors, signalling molecules, cytokines or other factors help test the role of these genes in immunologic mechanisms of arthritis. In addition, spontaneous inflammation sometimes occurs, which not only provides another model for the study of arthritis, but can help provide insights into immune regulation and mechanisms of autoimmunity (Kannan *et al.*, 2005). For example, mice with altered cytokine formation or responsiveness, including overexpression of human TNFα or IL1 (Keffer *et al.*, 1991; Kontoyiannis *et al.*, 1999; Niki *et al.*, 2001) or a deficiency of IL1Ra

(Horai *et al.*, 2000) can result in the development of arthritis in mice. In addition, there is a group of mice that spontaneously develop chronic arthritis as a result of a single gene mutation. An example of this includes mice with mutations at the Fas gene, although it is still unknown how altering a gene which impairs activation of induced cell death causes arthritis (Sakaguchi and Sakaguchi, 2005).

Work detailed in later chapters of this thesis will cover pain and nociception. The next section will therefore give a background to research in this field, and go on to describe peripheral and central mechanisms, as well as key mediators involved in inflammatory pain.

1.6 PAIN AND NOCICEPTION

The sensation of pain helps an organism detect potentially damaging external stimuli and avoid tissue damage from these stimuli (Sherrington, 1906). In addition, pain encourages an organism to protect the damaged area to aid repair and avoid further damage. A century ago Sherrington (1906) proposed there were specialized receptors to detect painful (noxious) stimuli in cutaneous tissue (Sherrington, 1906). From Sherrington's work the terms **nociceptors** and **nociception** eventually emerged (Burgess & Perl, 1967). According to Sherrington's model, nociceptors have characteristic thresholds or sensitivities that distinguish them from other sensory nerve fibres (Sherrington, 1906).

However, the term pain not only includes the neural sensation of noxious stimuli, but also the CNS's subjective perception of the sensation, and therefore pain experiences differ drastically among humans. Furthermore, we cannot assume that non-human animals experience the same "pain" that we do, merely that they experience nociception.

1.6.1 DISCOVERY AND CLASSIFICATION OF NOCICEPTORS

Early psychophysicists provided the first evidence that pain was a distinct sense from touch and was mediated by specialized sensory organs (von Frey, 1894; von Frey, 1922). Electrophysiological studies demonstrated the existence of primary sensory neurons that can be excited by noxious heat, cold, pressure or irritant chemicals, but not by innocuous stimuli such as warming or light touch (Burgess & Perl, 1967). Furthermore, it was observed that a variety of sensory nerves innervate peripheral tissues, with different functions. It was shown that thin myelinated (Aδ fibres) and unmyelinated axons (C-fibres) were mostly activated by noxious stimuli, whereas the largest myelinated fibres (Aβ-fibres) responded to innocuous stimuli (Clarke *et al.*, 1935; Gasser & Erlanger, 1927; Heinbecker *et al.*, 1932; Zotterman, 1933). However, asubstantial proportion of cutaneous and somatic afferent A-fire nociceptors conduct in the Aβ-fibre conduction velocity range (for review see Djouhri and Lawson, 2004). Recently, Djouhri *et al.* (2006) demonstrated that spontaneous pain arising in inflammatory and neuopathic pain models is related to the rate of spontaneous neural discharge of c-fibres. C-fibres have small fibre diameter (0.4-2 μm), Aδ-fibres having medium fibre diameter (2-6 μm), and Aβ fibres have the largest diameter (>10 μm; Millan, 1999). The conduction velocities of the different types of nerve fibre differ: C-fibres conduct at less than $2ms^{-1}$; Aδ fibres between 3 and 30 ms^{-1} and Aβ between 35 and 100 ms^{-1} (Erlanger & Gasser, 1937). Myelin is an electrically insulating phospholipid layer that surrounds the axons of many neurons. The main consequence of a myelin layer (or sheath) is an increase in the speed at which impulses propagate along the myelinated fibre. Along unmyelinated fibres, impulses move continuously as waves, in contrast, in myelinated fibres they propagate by saltation.

In the naïve knee joint the above fibres have been further classified into 4 groups according to their responsiveness to mechanical stimulation (Schaible & Schmidt, 1983b):

- Group I: nerves excited by innocuous movement.
- Group II: nerves weakly excited by innocuous movement while noxious movements lead to pronounced neural discharge.
- Group III: nerves that respond consistently only to noxious joint movements.
- Group IV: nerves that cannot be excited by any joint movement.

Approximately 70% of Aδ and C-fibres belong to groups III and IV (Schaible & Grubb, 1993).

Another study used anatomical and histological methods to study the innervation of 41 cat knee joints, and classified the articular nerve endings into four groups (Freeman & Wyke, 1967):

- Type 1 endings correspond to those previously described as Ruffini endings – slowly adapting cutaneous mechanoreceptors
- Type II endings were regarded as modified Pacinian corpuscles – rapidly adapting mechanoreceptors found in the mesenteries and near joints
- Type III endings were those with the description of Golgi tendon organs – a proprioceptive sensory organ located at the insertion of skeletal muscle fibres into the tendons of skeletal muscle
- Type IV endings were believed to be a system of free nerve endings and plexuses for nociception – uncapsulated, unspecialized, afferent nerve endings frequently found in the skin – responsible for detecting temperature, mechanical stimuli (such as pressure), nociception and information regarding touch.

However, the main criteria used to classify nociceptors is the stimulus that activates them; Bessou and Perl (1969) reported the response characteristics of a population of C-fibres innervating skin on the hind limb of a cat, they showed that approximately half of

these were activated by innocuous mechanical stimulation. The other 50%, the nociceptors, can be divided into 2 groups, 80% of which were activated by intense mechanical stimulation, high temperatures and chemical stimulation, these were called **polymodal nociceptors**. The remaining 20% responded only to noxious mechanical stimulation, these were called **mechanonociceptors** (Heppelmann, 1997; Heppelmann *et al.*, 1988a; Heppelmann *et al.*, 1988b; Hildebrand *et al.*, 1991).

A further classification system for nociceptive afferents includes molecules expressed on the cell surface, molecules stored and those released from nociceptive afferents. The expression of receptors for neurotrophic factors is of particular interest, as these factors may regulate the sensitivity of nociceptive afferents in adult animals (Bennett *et al.*, 1998a). Unmyelinated nociceptors fall into 2 groups, one of which expresses peptides including substance P and calcitonin gene related peptide (CGRP; 50% of C fibres; 20% of Aδ fibres). This group mediates the neurogenic inflammation induced by small vasoactive peptides, either directly or indirectly via mast cell degranulation and the subsequent release of histamine. The second group is nonpeptidergic, i.e. does not express substance P or CGRP (see Figure 1.4). Furthermore, non-peptidergic neurons and axons are characterised by their binding of the plant lectin IB4 from *Griffonia simplicifolia* (Silverman & Kruger, 1988). Most peptidergic neurons express the tyrosine kinase receptor A (TrkA), suggesting that they depend on nerve growth factor (NGF) for survival (Averill *et al.*, 1995). In contrast, most IB4-positive dorsal root ganglion (DRG) cells do not express TrkA (Molliver *et al.*, 1995), but express one of the glial cell line-derived neurotrophic factor family receptors (GFRα1-4) together with receptor tyrosine kinase RET (Bennett *et al.*, 1998b; Orozco *et al.*, 2001). These nociceptors contain a distinctive phosphatase called thiamine monophosphatase or fluoride-resistant acid phosphatase (FRAP+; Knyihar-Csillik & Csillik, 1981). They also express a subset of purinergic receptors ($P2X_3$), which are activated by adenosine triphosphate (ATP), see Section 1.6.4 for more details; Willis and Coggeshall, 1991; Chen *et al.*, 1995; Molliver *et al.*, 1995). Another unique feature of nociceptors in this

group is a high density of tetrodotoxin-resistant (TTX-r) sodium channels (Willis and Coggeshall, 1991). The third group is myelinated and therefore can be characterized based on its neurofilaments (NF200; Julius & McCleskey, 2006). These nociceptors contain CGRP and other peptides (Julius & McCleskey, 2006). Their membrane expresses receptors for neurotrophins belonging to the NGF family Transient Receptor Potential Vanilloid 2 receptor (TRPV2R; see Section 1.6.2; Julius & McCleskey, 2006).

A series of complex electrical and chemical events occurs between the occurrence of a noxious stimulus and the subjective experience of pain. These comprise four distinct processes: transduction, transmission, modulation and perception.

- *Transduction*, or receptor activation, is the process by which the external noxious stimulus is converted into electrophysiological activity in nociceptive primary afferent neurons.
- *Transmission* is the process by which this information is relayed to areas of the CNS concerned with pain. The first stage of transmission is the conduction of impulses along primary afferent neurons (first order neurons) to the dorsal horn of the spinal cord where they synapse onto second order neurons, intrinsic to the CNS. From here a network of neurons ascends the spinal cord to the brainstem and thalamus. Finally, connections are made with higher centres of the brain (third order neurons) concerned with the perceptive affective responses associated with pain. However, nociceptive activity does not always result in pain perception (equally, pain may be experienced in the absence of nociception); data from human microneurography experiments show that a single impulse or even low frequency discharge in a single nociceptive afferent appears to be insufficient to evoke a sensation of pain (Van Hees & Gybels, 1972).
- Therefore a process of signal *modulation* is needed if this system is capable of interfering in this pathway; the modulatory site about which most is known is the dorsal horn of the spinal cord.

- The final process is *perception*, in which the pain message is relayed to the brain, producing a subjective usually unpleasant sensory experience, which has affective, defensive and perceptive components (Beaulieu & Rice, 2002).

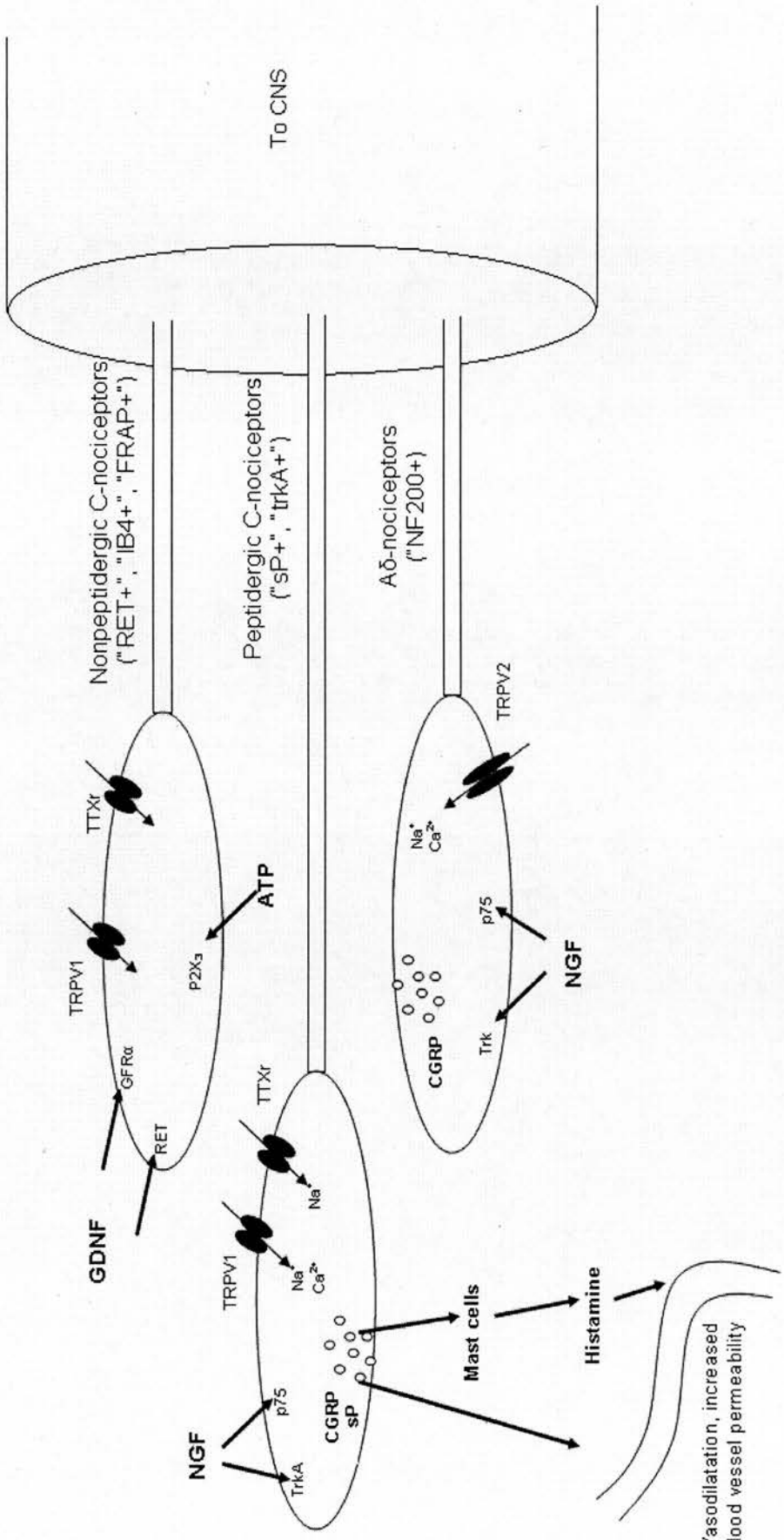


Figure 1.4. A schematic illustration of C- and Aδ-fibres. C-fibres are subdivided into peptidergic and nonpeptidergic fibres, which are selectively sensitive to NGF and GDNF respectively. Aδ-fibres are also sensitive to NGF (redrawn and adapted from Coutaux *et al*, 2005).

1.6.2 EXCITATION OF NOCICEPTORS

Noxious stimuli are detected when the peripheral terminals of nociceptive primary afferents are depolarised to the threshold for an action potential. The resting membrane potential of such fibres is approximately -70 mV in the normal state (Eccles & Krnjevic, 1959; Koketsu, 1956; Woodbury & Patton, 1952). Depolarisation occurs either as a result of an increased permeability of the membrane to ions such as sodium (Na^+) and calcium (Ca^{2+}), or a reduced permeability to potassium ions (K^+; Bevan, 1996b). The action potentials are transmitted along the axon to the CNS, where they may be perceived as pain, as described above.

Mechanical excitation

Mechanoreceptors that respond to low intensity mechanical stimulation or to normal joint movements have conduction velocities in the Aβ range (35 – 100 ms^{-1}). These sensors have proprioceptive functions which enable the position of the joint to be established as well as responding to vibration within the structure (Schaible & Schmidt, 1988). Movements beyond the normal range, or applied forces beyond certain thresholds are perceived as painful and these stimuli activate a separate population of Aδ-fibres, the **mechanonociceptors**. Furthermore, there are afferents which do not respond to innocuous or noxious movement, namely the "mechano-insensitive afferents" (Schaible & Grubb, 1993). These fibres do not appear to be important for encoding mechanical stimuli in the normal joint, but they may be required, or have altered function, during inflammation of the joint.

Among sensory modalities, mechano-sensation has been the most elusive with regard to the identification of molecules that mediate stimulus detection and transduction. Recent electrophysiological studies have shown primary sensory neurons from rat dorsal root or trigeminal ganglia retain sensitivity to mechanical stimuli when dissociated and

placed in culture; this has enabled experiments to show that mechanically sensitive DRG neurons can be divided into high and low threshold classes based on their responses to stimuli of graded intensity (Cho *et al.*, 2002; Drew *et al.*, 2002).

However, much of what is known has been from studies in non-mammalian systems, including the *Eschericia coli* mechano-sensitive channel of large conductance (MscL; reviewed by Sukharev *et al.*, 1997). Purified MscL proteins form functional mechano-sensitive channels when reconstituted into artificial lipid bilayers (Hase *et al.*, 1995) indicating that stretching the lipid bilayer directly opens the channel.

The genetic manipulation of *Caenorhabditis elegans* results in it being insensitive to touch (Tavernarakis & Driscoll, 1997). One of the genes required by *C.elegans* for mechano-sensitivity is thought to encode a mechano-sensitive ion channel homologous to the amiloride-sensitive, voltage-insenstive sodium channel; amiloride is well known to inhibit mechano-sensitive ion channels in mammalian systems (Hamill *et al.*, 1992; Kellenberger and Schild, 2002). Acid-sensing ion channels (ASIC) represent a H+-gated subgroup of the degenerin/epithelial Na+ channel family of cation channels, are thought to be involved in mechanotransduction, but more work is required to fully determine their role in this process (for review see Wemmie *et al.*, 2006).

Using frog oocytes as a model system, it has been shown that mechanical stimulation can release ATP from the cell, promoting autocrine activation of P2Y receptors on the cell's surface. *In vivo*, mechanical force might therefore promote ATP release from one or more cell types in the periphery, where it could activate purinergic receptors on nearby nociceptor terminals (Nakamura & Strittmatter, 1996). Furthermore, a cRNA derived from sensory neurons which renders *Xenopus* oocytes mechanosensitive was found to encode a $P2Y_1$ receptor (for review see Burnstock *et al.*, 2000).

In addition, a member of the TRP family of receptors, TRPA1, has been proposed as a candidate for mechanotransduction. *In vitro* knockdown studies have shown that TRPA1 was a promising candidate for the mechanosensitive transduction channel of hair cells in the inner ear (Corey *et al.*, 2004). OSM-9 and OCR2 are members of the TRP family needed for avoidance of nose touch and high osmolarity and are coexpressed in ciliary mechanosensory endings (Colbert *et al.*, 1997; Tobin *et al.*, 2002). No mechanoreceptor potential C (NOMPC) mediates rapidly adapting receptor currents in bristle mechanoreceptor epithelia and is expressed in *C.*elegans mechanosensory neurons that detect variations in substrate texture (Sawin *et al.*, 2000; Walker *et al.*, 2000).

There are now several more proteins with potential roles in mechanotransduction (for review see Goodman *et al.*, 2004) and further investigation of these receptors or mice lacking their expression will increase knowledge of the transduction mechanisms, but for now, the exact characteristics of peripheral mechanonociceptors remain elusive.

Thermal excitation

Sensory neurons are ale to detect a wide range of temperatures. Temperatures outside the normal physiological range are perceived as painful, these signals are transmitted by **thermanociceptors**.

Noxious heat

In sensory ganglia dissociated in culture, approximately 45% of small- to medium-diameter neurons exhibit heat evoked membrane currents with a "moderate" threshold of 45°C, whereas another 5 – 10% of cells respond with a "high" threshold of ~52°C (Caterina *et al.*, 1997; Cesare & McNaughton, 1996; Nagy & Rang, 1999). The former corresponds to C and type II Aδ nociceptors, and the latter to type I Aδ nociceptors. Recent studies suggest that this is mediated via a cation selective ion channel that is also gated by capsaicin the selective ingredient of chilli peppers.

In the mid-19th century, the principal pungent component of peppers of the genus *Capsicum* was isolated and named capsaicin (Thresh, 1946). Several decades later, it was proposed that capsaicin acted selectively on sensory neurons, resulting in the sensation of pain. The structure of capsaicin was revealed as an acylamide derivative of homovanillic acid, 8-methyl-*N*-vanillyl-6-noneamide (Nelson, 1991). Later, it was demonstrated that capsaicin was capable of preventing the sensation of painful stimuli in animals, as well as activating sensory neurons (Jancso *et al.*, 1967; Szolcsanyi *et al.*, 1988). Capsaicin has been a valuable tool, as a functional marker for nociceptors. Studies of capsaicin action have provided insights into the activation of primary afferent nociceptors and have revealed the efferent role of some nociceptors, acting to evoke inflammation or smooth muscle contraction (Maggi, 1995; Szolcsanyi, 1996). Moreover, an appreciation of the mechanisms by which capsaicin desensitises neurons has provided a rational basis for the use of capsaicin and related compounds, such as resiniferatoxin, in the treatment of painful disorders ranging from diabetic neuropathy to arthritis, although use of an antagonist to the receptor would be more beneficial.

Genetic methods have been used to assess the roles of the TRPV1R in thermo- and chemo-nociception (Caterina *et al.*, 2000; Davis *et al.*, 2000). Mice bearing targeted deletions within the TRPV1 gene were examined for thermal sensitivity at numerous levels of the pain pathway, including cultured sensory neurons, primary afferent fibres (skin-nerve preparation), spinal cord dorsal horn neurons and animal behaviour. At each level, significant deficits were observed in heat sensitivity. However, it is clear that these mice are not completely insensitive to thermal nociception suggesting that TRPV1 mediates some, but not all, aspects of thermal nociception. It has been hypothesised that the remaining thermal sensation is mediated by another member of the TRP family (Julius, 2004). TRPV2 has 50% homology to TRPV1, but it has no capsaicin binding site (Caterina *et al.*, 1999). It is sensitive to heat >50°C and is probably expressed in high-threshold heat-sensitive small

myelinated nociceptors units (Aδ; Caterina *et al.*, 1999; Lewinter *et al.*, 2004; Ma, 2001). The discovery and cloning of the TRPV1 channel revealed that it was sensitive to not only capsaicin, but also high temperatures (>42°C) and protons, pH is <6 (Caterina *et al.*, 1997).

Hyperalgesic neural responses, such as *c-fos* expression in the dorsal horn of the spinal cord induced by inflammation, are blocked by capsazepine (Kwak *et al.*, 1998), a TRPV1 antagonist. However, capsazepine at micromolar concentrations, which are necessary to inhibit capsaicin-evoked responses in most tissues, also blocks voltage-dependent calcium channels (Docherty *et al.*, 1997; Kuenzi & Dale, 1996), voltage-dependent potassium channels (Kuenzi & Dale, 1996) as well as nicotinic acetylcholine receptors (Liu & Simon, 1997).

It has been speculated that other than voltage and protons, an endogenous ligand for TRPV1 may exist. Three different classes of lipids, all derived from the metabolism of arachidonic acid, have recently been characterised that can activate TRPV1; these are the endocannabinoid anandamide, some lipoxygenase products of arachidonic acid, and N-arachidonoyldopamine. The levels of endocannabinoids are elevated in pain (Rice *et al.*, 2002; Walker & Huang, 2002) an observation that has led to speculation that they play an important role in the regulation of pain processing and nociceptive neuronal excitability in pathophysiological situations.

The endogenous cannabinoid receptor agonist anandamide is a powerful vasodilator when studied in isolated vascular preparations. Anandamide-evoked vasodilatation is capsaicin-sensitive and accompanied by release of CGRP (Zygmunt *et al.*, 1999). The selective CGRP-receptor antagonist 8-37 CGRP, but not the cannabinoid (CB) 1 receptor antagonist SR141716A, inhibited the vasodilator effect of anandamide. Other endogenous (2-arachidonylglycerol, palmitylethanolamide) and synthetic (HU 210, WIN 55,212-2, CP 55,940) CB1 and CB2 receptor agonists could not mimic the action of anandamide. The

vanilloid receptor antagonist capsazepine, inhibited anandamide-induced vasodilatation and release of CGRP. Moreover, in patch-clamp experiments on cells expressing TRPV1, anandamide induced a capsazepine-sensitive current in whole cells and isolated membrane patches (Zygmunt *et al.*, 1999).

Products of lipoxygenase enzymes are able to activate TRPV1 (Hwang *et al.*, 2000). These are released during inflammation (Samuelsson, 1983) and cause hyperalgesia when injected intra-dermally in the rat paw (Levine *et al.*, 1984). In addition, products of lipoxygenase activity often function as intracellular second messengers in neurons (Kim & Oh, 2004). 12- hydroperoxy eicosatetraenoic acids (HPETE) activated single-channel current that were blocked by capsezipine in isolated membrane patches. Furthermore, the current-voltage curve of a single-channel currents activated by 12-HPETE is outwardly rectifying, identical to that obtained with capsaicin (Kim & Oh, 2004). Other products of lipoxygenase also activate TRPV1, including 15-HPETE, 5- and 15-hydroxyeicosatetraenoic acids (5- and 15- HETEs) and leukotriene (LT) B4 (Kim & Oh, 2004). PGs sensitise nociceptive fibres, which suggest a possible effect on TRPV1, but they fail to activate the channel (Kim & Oh, 2004).

There are receptors in the skin that respond to mild changes in temperature. Warm receptors are predominantly found on unmyelinated fibres, are highly sensitive to gentle warming to their punctuate receptive fields. These fibres have been shown to signal exclusively the quality and intensity of warmth sensation (Johnson *et al.*, 1979). Another member of the TRP family of receptors responsible for this warm sensation is TRPV3 (33°C threshold; Peier *et al.*, 2002b; Smith *et al.*, 2002; Xu *et al.*, 2002). Warm receptors conduct almost exclusively in the C-fibre range and are also spontaneously active at normal skin temperature and show linear increase in discharge as skin temperature increases up to 46°C. Outside these constraints the afferents become silent (Darian-Smith & Johnson, 1977).

Noxious cold

Compared with our understanding of noxious heat sensation, less is known about cold receptors. Depending on the study and experimental system, approximately 10-15% of C and Aδ fibres respond to application of cold stimulus (Bessou & Perl, 1969; Hensel & Zotterman, 1951; Kress *et al.*, 1992). Furthermore, it was noted that cultured sensory neurons produced responses to cold or menthol application with properties similar to those of the TRPV1 channel, this new channel was cloned and termed the cold- and menthol-sensitive channel (CMR1 or Transient Receptor Potential Menthol 8; TRPM8; McKemy *et al.*, 2002; Peier *et al.*, 2002a; for review see Dhaka *et al.*, 2006 or Reid, 2005). TRPM8 is activated by moderate to strong cooling from 28° C to 8° C, independent of the rate of direction of temperature change (Handwerker, 2006; McKemy *et al.*, 2002). Studies of cold or menthol responses in nerve fibres in cultured sensory neurons have produced several models to explain how cold and menthol promote neuronal depolarization, including inhibition of background (or leak conductance's – a major determinant of membrane resting potential) potassium channels, inhibition of Na^+/K^+ ATPases, activation of Na^+-selective epithelial sodium channel/ degenerin channels, and activation of Ca^{2+} permeable channels (Askwith *et al.*, 2001; Braun *et al.*, 1980; Pierau *et al.*, 1974; Reid & Flonta, 2001a; Reid & Flonta, 2001b; Suto & Gotoh, 1999).

A second novel cold-activated TRP channel has been characterised, TRPA1 (17°C threshold; activated at a broad range of temperatures 12°C - 24°C ;Peier *et al.*, 2002a; Story *et al.*, 2003). TRPA1 is expressed in either Aδ- and/or C-fibres; it is coexpressed with CGRP, substance P and TRPV1, all of which is consistent with a role in cold nociception (for review see Dhaka *et al.*, 2006). TRPA1 bears little homology to TRPM8 and has no menthol binding site.

Chemical excitation

Nociceptors can be activated by some chemical irritants acting via specific pharmacological receptors at their terminals to produce an acutely painful response. During inflammation, in inflamed tissues, a variety of chemical mediators are released that are able to directly or indirectly activate primary afferent nociceptors leading to more chronic pain. The mechanism by which inflammatory agents excite or sensitise nociceptors is described in Section 1.6.4 in more detail.

1.6.3 NOCICEPTORS INNERVATING THE NORMAL AND INFLAMED KNEE JOINT

There is extensive literature on sensory nerve innervations in a wide variety of tissues throughout the body including skin (Bessou & Perl, 1969; Torebjork & Hallin, 1974), joints (Heppelmann, 1997; Mapp, 1995; Schaible & Schmidt, 1983a), cornea (Belmonte *et al.*, 1991; Belmonte & Giraldez, 1981; Giraldez *et al.*, 1979), skeletal muscles (Mense & Meyer, 1985), airways (Fox *et al.*, 1993), viscera (Cervero, 1994) and teeth (Greenwood *et al.*, 1972). Only the articular afferent innervation of the knee joint, in particular nociceptors, will be discussed further, due to the relevance to experimental work carried out.

Early comprehensive studies of the anatomy of the knee joint were carried out in cats (Freeman & Wyke, 1967; Gardner, 1944; Skoglund, 1956). More recently, electron microscope studies have provided a detailed morphological analysis of the nociceptive afferent endings innervating the joint (Heppelmann *et al.*, 1988a). Although the cat knee joint has been the most extensively studied and documented, studies examining the innervation of other species' knee joints have also been carried out, including rats (Hildebrand *et al.*, 1991) humans (Biedert *et al.*, 1992; Halata *et al.*, 1985) and mice (Ebinger *et al.*, 2001). These studies have shown that the pattern of innervation, the types of fibres and the ratio of myelinated to unmyelinated fibres are very similar between species.

The most notable difference between the species was the absolute number of fibres innervating the joint, but this is dependent on animal size.

The articular branches that exit the saphenous nerve on the medial aspect of the limb supply the knee joint. The knee joint innervation arises primarily from two articular nerves, the posterior articular nerve (PAN) and the medial articular nerve (MAN). Electrophysiological recordings during experimental work in this thesis record from the MAN, therefore further discussions will focus on this articular nerve.

The MAN branches from the saphenous nerve and traverses the antro-medial aspect of the thigh and runs alongside the descending genicular artery and vein, to the medial aspect of the knee joint. At this point the nerve divides into two branches and spreads out to innervate the medial and antero-medial aspects of the fibrous capsule of the knee joint, the medial collateral ligament, the medial part of the annular ligament attached to the medial meniscus, the ligamentum patellae and the infrapatellar fat pad , and the medial part of the patellar periosteum (Freeman & Wyke, 1967).

The MAN of the cat contains approximately 630 afferent and 500 unmyelinated sympathetic efferent fibres. Of the afferents the majority are Aδ- and C-fibres (groups III and IV; 20% and 70% respectively). The remaining are Aβ-fibres (Langford & Schmidt, 1983). Approximately 60% of knee joint innervation may be classified as nociceptive (Heppelmann, 1997). In the rat, a large PAN and a small MAN can be identified. The PAN is composed of approximately 400 axons, about 80% of which are unmyelinated. All myelinated fibres are sensory; they range in size between 1-8 μm. Approximately one-third of the unmyelinated axons represent afferents (Hildebrand *et al.*, 1991).

From their peripheral terminals, articular afferents extend long axons up to the cell bodies in the DRG.

In addition to sending axons to the periphery, articular nociceptors project axons from the DRG into the dorsal horn of the spinal cord. Using retrograde labelling of axons in the cat it has been shown that afferents of the MAN enter the spinal cord via L5 and L6

(Craig *et al.*, 1988; Skoglund, 1956). Once in the dorsal horn these afferents have projections into different layers of laminae.

1.6.4 INFLAMMATORY PAIN

An injury or site of inflammation can heighten the level of pain experienced by increasing the sensitivity of nociceptors to thermal, mechanical and chemical stimuli. This is partly a result of the production and release of chemical mediators into the micro-environment from primary sensory terminals and non-neuronal cells, including immune cells and platelets (Bevan, 1999). These mediators either directly activate nociceptors or sensitise the primary afferents to other stimuli.

Due to the excitatory and sensitising effects of inflammatory mediators, nociceptors innervating inflamed tissues have an increased basal rate of discharge, a reduced threshold for activation and an augmented response to stimuli. Furthermore, during inflammation more nociceptors are excited by any given stimulus and nociceptors that were previously inactive are now activated (Coggeshall *et al.*, 1983; Guilbaud *et al.*, 1985; Schaible & Schmidt, 1985; Schaible & Schmidt, 1988).

There are two phenomena that occur in inflammatory conditions, and the nomenclature used here will be that defined by the International Association for the Study of Pain (IASP) subcommittee on taxonomy; **hyperalgesia** is 'an increased response to a stimulus that is normally painful and should not be associated with a lowering of threshold'; whereas **allodynia** is commonly used when referring to 'pain due to a stimulus that does not normally produce pain' (Merskey & Bogduk, 1994; see Figure 1.5). These enhanced pain states arise from both peripheral and central mechanisms of altered pain transmission.

Hyperalgesia exists both at the site of injury and in the uninjured surrounding tissue, these are known as **primary** and **secondary hyperalgesia** respectively (Lewis, 1942). Secondary hyperalgesia can be partly explained by enhanced synaptic responses of second-

order neurons in the spinal cord to their normal afferent input (central sensitisation; Koltzenburg, 2000; LaMotte *et al.*, 1991; Torebjork *et al.*, 1992). However, there are also indications that peripheral mechanisms play a role, including the axon reflex (Serra *et al.*, 1998). Local axon reflexes depolarise other branches of the same sensory axon and result in more widespread release of inflammatory mediators, this is mediated by peptidergic C-fibres, and includes enhanced pain from mechanical but not heat stimuli (Ali *et al.*, 1996). Multiple intracutaneous injections of lidocaine after a nearby capsaicin injection block the spreading of the axon reflex and also the development of punctuate hyperalgesia (LaMotte *et al.*, 1991; Serra *et al.*, 1998).

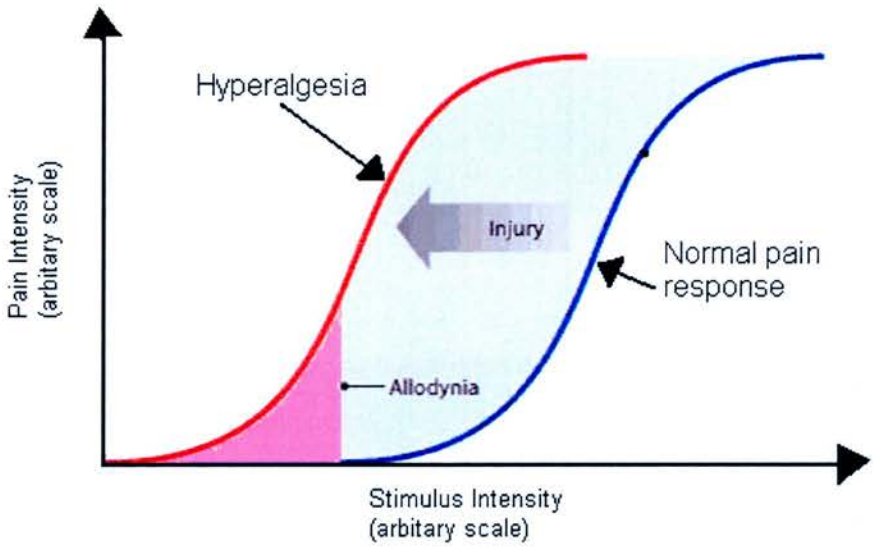


Figure 1.5. Noxious stimuli can sensitise the neural response to subsequent stimuli. The normal pain response as a function of stimulus intensity is depicted by the curve on the right, where even strong stimuli are not experienced as pain. However, a traumatic injury can shift the curve to the left. Then, noxious stimuli become more painful (hyperalgesia) and typically painless stimuli are experienced as pain (allodynia; illustration redrawn and adapted from Gottschalk & Smith, 2001).

Peripheral mechanisms

Injury or inflammation results in the local release of chemicals that mediate or facilitate the inflammatory process, including bradykinin (BK), PGs, LTs, 5-hydroxytryptamine (5-HT; serotonin), histamine, substance P, thromboxanes, PAF, adenosine, ATP, protons and free radicals. Cytokines, such as IL1 and TNF, and neurotrophins, especially NGF, are also generated (see Figure 1.6). Some of these agents

can directly activate nociceptors, while others act indirectly via inflammatory cells, which in turn, release algogenic agents. Other mediators lead to a sensitisation of the nociceptor's response to natural stimuli and therefore play a role in primary hyperalgesia.

Bradykinin (BK)

Several lines of evidence suggest BK plays a critical role in inflammatory pain (for review see Dray, 1997). BK is released as a result of tissue injury and is present in inflammatory exudates. In addition, it has been shown to evoke pain in humans when administered intradermally, intra-arterially, intravenously or intraperitoneally. Furthermore, administration of BK in the region of the receptive field of unmyelinated and myelinated nociceptors results in an evoked response in these fibres (Beck & Handwerker, 1974), however, this response undergoes tachyphylaxis upon repeated applications. BK not only activates nociceptors, but also sensitises them to heat stimuli (Khan *et al.*, 1992; Lang *et al.*, 1990), however, unlike the evoked response, the sensitisation does not undergo tachyphylaxis (Manning *et al.*, 1991).

This BK-induced excitation in inflamed rat skin is thought to occur through B2 receptors, since a B2-receptor antagonist blocks the effect on C-fibre nociceptors, while B1-receptor antagonists have no effect (Banik *et al.*, 2001). However, conflicting reports suggest that B1-receptors do play a role in BK-induced afferent sensitisation (Reeh & Sauer, 1997). BK receptors are coupled to guanyl-nucleotide binding proteins (GPCR) and result in a second messenger signalling cascade. The BK B_2 receptor activation pathway on sensory neurons results in the activation of PLC which cleaves membrane lipids to form inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG activates protein kinase-C (PKC) which can phosphoylate membrane ion channels, including TRPV1 and receptors leading to membrane depolarisation (Bevan, 1996a).

5-hydroxytriptamine (5-HT; Serotonin)

Mast cell degranulation releases PAF, which evokes 5-HT release from platelets. 5-HT rapidly depolarises the cell membrane by binding to specific ligand-gated ion-channel linked receptors. 5-HT causes pain when applied to a human blister base (Richardson & Engel, 1986), when injected into human skin (Schmelz *et al.*, 2003) and can activate nociceptors in the rat (Lang *et al.*, 1990) and a population of mechanically-insensitive afferents (Schmelz *et al.*, 2003). Furthermore, 5-HT can potentiate the pain induced by BK and hence the response of nociceptors to BK. In addition, it has been shown to excite and sensitise group III and IV afferents to mechanical stimulation in the cat knee joint and the rat ankle joint (Birrell *et al.*, 1990; Herbert & Schmidt, 1992). There are many subtypes of 5-HT receptors and several of these are implicated in pain (for review see Zemlan *et al.*, 1988).

Protons

The pH of the surrounding skin tissue decreases following skin incision, (Woo *et al.*, 2004). The protons responsible for the drop in pH may play a role in activating and sensitising nociceptors. Pain and hyperalgesia to mechanical stimuli are produced in humans following continuous cutaneous administration of low pH solutions (Steen & Reeh, 1993) and this pain is potentiated by co-administration of low pH with inflammatory mediators in humans (Steen *et al.*, 1996). This correlates with the observation that protons selectively activate nociceptors and produce sensitisation of nociceptors to mechanical stimuli. (Steen *et al.*, 1996; Steen *et al.*, 1995).

Specific channels have been identified that respond to protons. In the DRG, ASIC 3 mediates acid-evoked pain and hyperalgesia. ASIC receptors have been implicated as the major mediators of proton-induced cutaneous pain in humans (Jones *et al.*, 2004). However, the TRPV1 channel in nociceptors is also responsive to protons (McLatchie & Bevan, 2001; Tominaga *et al.*, 1998).

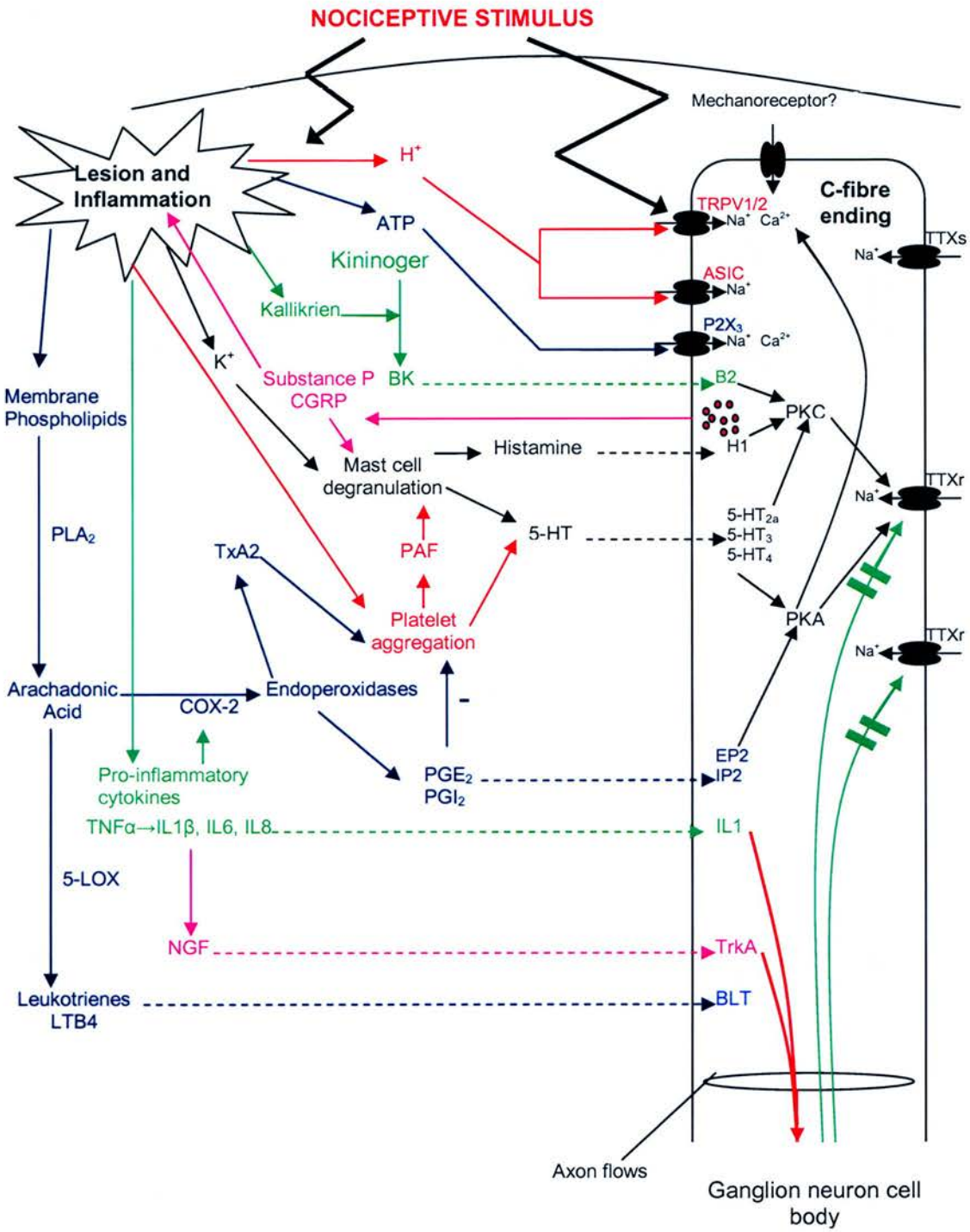


Figure 1.6. Receptors, nociception and inflammation. This figure shows the mediators that activate (→) and/or sensitise (--->) nociceptors at sites of tissue damage. Redrawn and adapted from Coutaux *et al.*, 2005).

Histamine

During tissue injury, mast cells can release histamine when activated by substance P from nociceptor terminals. Histamine can lead to a variety of responses including vasodilatation and oedema. Furthermore, histamine application causes excitation of nociceptors. Canine polymodal visceral receptors *in vitro* are excited by histamine at high concentrations (Koda *et al.*, 1996) and histamine potentiates the response of nociceptors to BK and heat (Mizumura *et al.*, 1995), suggesting that under conditions of inflammation histamine may play a role in the production of hyperalgesia. Mechanosensitive cutaneous nociceptors in rat and humans respond only weakly to histamine (Lang *et al.*, 1990). However, a subpopulation of mechanosensitive C fibres is vigorously excited by histamine in human microneurography experiments (Schmelz *et al.*, 1997). However, application of histamine to human skin produces a sensation of itch rather than pain (Simone *et al.*, 1991), and intradermal injection of histamine in mice is used to induce itch (Bell *et al.*, 2004).

Arachidonic acid metabolites

Arachidonic acid is metabolised into a number of compounds collectively known as eicosanoids: PGs, thromboxanes (Txs) and LTs. The eicosanoids are generally not thought to activate nociceptors directly, but instead they sensitise nociceptors in skin and viscera to heat, mechanical stimuli and other endogenus chemicals (for reviews see Cunha & Ferreira, 2003; Schaible *et al.*, 2002). However, there are exceptions, including PGE_2 and prostacyclin (PGI_2), which have been shown to directly excite as well as sensitise joint afferent nociceptors (Birrell *et al.*, 1991; Grubb *et al.*, 1991; Heppelmann *et al.*, 1986; Schaible & Schmidt, 1988). Furthermore, PGE_2 activates C-fibre mechano- heat- sensitive nociceptors and a subpopulation of mechano-insensitive afferents (Schmelz *et al.*, 2003). PGI_2 has a much greater role in sensitisation of nociceptors; in the rat ankle joint PGI_2 and the more stable agonist cicaprost sensitised the majority of nociceptors (80-90%) to mechanical stimulation (Birrell *et al.*, 1991). PGE_2 and PGI_2 act on specific cell surface

receptors to increase AC activity intracellularly. AC mediated increases in intracellular cyclic adenosine mono-phosphate (cAMP) have been implicated in nociceptor sensitisation (Levine & Tawoi, 1994). PGs reduce the threshold for activation of voltage-sensitive TTX-r sodium current specific nociceptors, increase intracellular cAMP and increase the excitability of sensory neurons (Bevan, 1996a; England *et al.*, 1996; Gold *et al.*, 1996). PGs are synthesized by the constitutively expressed COX-1 and by the inducible COX-2, which is increased during inflammatory conditions (Ballou *et al.*, 2000).

LT D4 and B4, produced by lipoxygenase enzymes, may play a role in producing mechanical hyperalgesia (Levine *et al.*, 1984) and in sensitisation to mechanical stimuli (Martin *et al.*, 1987).

Adenosine and adenosine phosphates

During inflammation and tissue injury, adenosine, adenosine monophosphate, adenosine diphosphate and ATP are released into the extracellular space and activate nociceptors. Adenosine and its phosphates have been reported to induce pain when applied to a human blister base or are administered by intra-arterial or intradermal injections (Bleehen & Keele, 1977). The adenosine receptor family comprises four subtypes: A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al.*, 2001). In animals, adenosine enhances the response to formalin, via the A_2 receptor. Animals lacking the A_2 receptor are hypoalgesic to heat stimuli (Ledent *et al.*, 1997). The role of adenosine receptors in nociception is complex and may involve different mechanisms in the CNS and in peripheral tissues. For example, spinal administration of adenosine receptor agonists produces antinociception in a variety of animal models of pain through the activation of spinal A_1 and to a lesser extent through A_2 receptors (Holmgren *et al.*, 1986; Sawynok, 1998). Adenosine can produce analgesic or pronociceptive effects (Doak & Sawynok, 1995) through activation of A1 and A2 receptors respectively (Doak & Sawynok, 1995; Tawoi & Levine, 1990).

A number of lines of evidence suggest a role of ATP as a peripheral mediator of pain. Increased levels of ATP are found at sites of inflammation and can activate nociceptors. ATP-induced pain is dependent on capsaicin-sensitive neurons and repeated topical application of capsaicin reduces the ATP-induced pain. In human microneurography experiments, injection of ATP in the receptive field of nociceptors results in responses in more than 60% of mechano-responsive and mechanic-insensitive C fibres. However, ATP does not sensitise the C fibres to mechanical or heat stimuli (Hilliges *et al.*, 2002). It has been found that ATP acts directly on the terminals of peripheral sensory nerves (Dowd *et al.*, 1998), through what is thought to be a receptor complex made up of $P2X_2$ and $P2X_3$ subtypes (Lewis *et al.*, 1995). Moreover, knockout mice lacking the $P2X_3$ receptor, showed a modest reduction in hind paw licking and lifting after intraplantar formalin (Cockayne *et al.*, 2000).

Receptors for ATP are found in both the DRG and the periphery and are composed of two major classes, the P2X (ligand-gated cation channels) and P2Y groups (GPCR) (Abbracchio & Burnstock, 1994). $P2X_3$ and $P2X_{2/3}$ have very restricted distribution patterns; they are only expressed in the cell bodies of thin myelinated and unmyelinated sensory afferents (Chen *et al.*, 1995; Lewis *et al.*, 1995). Dose-dependent pain behaviour is observed when P2X agents are intradermally injected in rodents (Bland-Ward & Humphrey, 1997), and enhanced pain behaviours to formalin are seen in rats (Sawynok & Reid, 1997). Administration of a P2X antagonist reversed the mechanical hyperalgesia produced by inflammation. Furthermore, the C-fibre sensitisation produced by a P2X agonist is blocked by a P2X antagonist (Wu *et al.*, 2004). In addition, the $P2X_7$ receptor is present on mast cells, which during inflammation release a large number of pro-LTs.

Excitatory amino acids

Evidence exists that suggest a role of peripheral metabotropic glutamate receptors in nociception and inflammatory pain (see Carlton, 2001). Peripheral application of glutamate

activates nociceptors, and peripheral administration of ligands binding to glutamate receptors induces pain behaviour in animals. For example, intraplantar injection of glutamate in the rat hind paw produces mechanical hyperalgesia (Carlton *et al.*, 1995). A subsequent study demonstrated that intraplantar injection of the specific glutamate receptor agonists NMDA, AMPA or kainate results in mechanical hyperalgesia and allodynia that can be blocked by appropriate antagonists (Zhou *et al.*, 1996). Similarly, intra-articular injection of excitatory amino acids in the rat knee joint (Lawand *et al.*, 1997) or the tail (Carlton *et al.*, 1998) results in thermal hyperalgesia and mechanical allodynia that is attenuated by local administration of NMDA or non-NMDA receptor antagonists (Lawand *et al.*, 1997; Carlton *et al.*, 1998). Furthermore glutamate activates and sensitises nociceptors in rat glaborous skin (Du *et al.*, 2001). An involvement of peripheral glutamate receptors in formalin-induced pain behaviours and glutamate-induced thermal hyperalgesia has been demonstrated (Davidson *et al.*, 1997).

Endogenous sources of glutamate in the periphery include plasma, macrophages, epithelial cells and dendritic cells.. In addition, peripheral processes of the primary afferents contain glutamate, and nociceptor stimulation can cause the peripheral release of glutamate from the terminals of these afferents (deGroot *et al.,* 2000).

Acetylcholine

Non-neuronally released acetylcholine (ACh) acting on peripheral cholinergic receptors may have a modulatory role on nociception. Nicotine has a weak excitatory effect on C-fibre nociceptors, and induces a mild sensitisation to heat but no alterations in mechanical responsiveness. However, muscarine desensitises C-fibre nociceptors to mechanical and thermal stimuli (Bernardini *et al.*, 2001), therefore nicotinic and muscarinic receptors may have opposing actions on cutaneous nociceptors.

TRPV1 agonists

The vanilloid receptor, TRPV1 is expressed only in a minority of IB4-positive nerve cells in mice (Zwick *et al.*, 2002) and IB4-positive neurons are less responsive to heat, capsaicin and protons than their IB4-negative counterparts (Dirajlal *et al.*, 2003; Stucky & Lewin, 1999). In rat this distinction is less obvious, as about half of both IB4-positive and IB4-negative cells express TRPV1 (Caterina *et al.*, 1997; Guo *et al.*, 2001; Guo *et al.*, 1999; Michael & Priestley, 1999). Axonal transport of TRPV1 mRNA is induced by inflammation; the proportion of TRPV1-labelled unmyelinated axons in the periphery is increased by almost 100% (Carlton & Coggeshall, 2001) and the sensitivity of primary afferents to capsaicin increases (Tohda *et al.*, 2001). Studies using TRPV1 knockout mice show a crucial role for the receptor in thermal hyperalgesia and swelling (Caterina *et al.*, 2000; Keeble, 2004).

Neuropeptides

Activated peptidergic C-fibres release neuropeptides such as substance P and CGRP, which in turn cause local increases in blood flow and endothelial permeability (McMurdo *et al.*, 1997), leading to recruitment of more blood-borne immune cells to the site for tissue repair. The release of substance P and CGRP is not confined to the immediate vicinity of the injury because depolarised nerve endings activate local axon reflexes resulting in more widespread release of these mediators (Helyes *et al.*, 2003; Langley, 1921). CGRP and substance P released from sensory neurons contribute to further sensitisation of nerve endings via stimulation of production of prostaglandins by surrounding cells.

Voltage-gated sodium channels

Sodium channel blockers are powerful analgesics at low concentrations (Strichartz *et al.*, 2002). Two sodium channels, $Na_v1.8$ and Na_v 1.9 are selectively expressed within the peripheral nervous system, predominantly in nociceptive sensory neurons. Na_v 1.7 is found

in both sympathetic and nociceptive sensory neurons and has been shown to play a critical role in inflammatory pain using nociceptor-specific knockout mice (Nassar *et al.*, 2004). Studies of DRG neurons from mice lacking the $Na_v1.8$ gene indicate this channel accounts for almost all TTX-resistant current during action potentials in these neurons (Akopian *et al.*, 1999). Behavioural studies of these mice revealed a complete absence of responses to a tonic noxious mechanical stimulus and attenuated primary thermal hyperalgesia evoked by intraplantar injection of NGF (Akopian *et al.*, 1999; Kerr *et al.*, 2001). Inhibiting the expression of $Na_v1.8$ protein using antisense oligonucleotides also reduces primary hyperalgesia produced by intraplantar PGE_2 or FCA in rats (Khasar *et al.*, 1998; Porreca *et al.*, 1999).

Central Mechanisms

The dorsal horn of the medulla and spinal cord are the major sites of termination of nearly all sensory afferents. Central axons of primary afferents ascend or descend one or two segments in Lissauer's tract before terminating in specific regions of the grey matter of the spinal cord. The dorsal horn was divided into ten laminae based on histology (Rexed, 1952; for review see Willis and Coggeshall 1991). Unmyelinated C-fibre nociceptors terminate principally in lamina II (the subsantia gelatinosa; Willis and Coggeshall, 1991; Millan, 1999). Small myelinated Aδ nociceptors terminate mainly in the superficial dorsal horn (lamina I). Nociceptors from joint terminate in lamina I as well as more deeply in lamina VI and VII. Large fibre, low threshold mechanoceptors terminate mainly in lamina III and IV, or more rostrally in the dorsal column nuclei of the medulla oblongata (Todd & Koerber, 2006).

The terminations of primary afferent nociceptors transmit information to second order neurons in the dorsal horn. These can be divided into two classes, namely; nociceptor specific (Besson and Chaouch, 1987) or wide dynamic range neurons that have different

response properties to afferent input and differentiatial distributions in regions of the dorsal horn (Willis and Coggeshall, 1991).

Neurotransmission from the dorsal horn encompasses:

- Excitatory transmitters released from the central terminals of primary afferent nociceptors;
- Excitatory transmission between neurons of the spinal cord;
- Inhibitory transmitters released by interneurons within the spinal cord;
- Inhibitory transmitters released from supraspinal sources.

Glutamate is the main CNS neurotransmitter and plays a major role in nociceptive transmission in the dorsal horn. Glutamate acts at AMPA, NMDA and kaniate receptors. The release of substance P, which coexists in primary afferents with glutamate, occurs following cutaneous thermal, mechanical or chemincal noxious stimuli and is potentiated by peripheral inflammation.

The increased C-fibre activity during inflammation increases the excitability of second order spinal neurons via release of glutamate and substance P. Experimentally, repeated stimulation of primary afferent C-fibres above a threshold rate induces a cumulative increase in the action potential firing of dorsal horn neurons *in vivo* (Mendell, 1966). This is called **wind-up**, which effectively increases the gain of afferent inputs (Kress & Reeh, 1996). In addition, brief high frequency stimulation induces a long-term increase in excitatory synaptic transmission onto superficial dorsal horn neurons which outlasts the period of stimulation. This strengthens the efficacy of synaptic transmission, in a similar way to long-term potentiation of the hippocampus (Ji *et al.*, 2003), and is called **central sensitisation**.

Some work has also shown that following peripheral nerve injury the central terminals of peripheral Aβ-fibres sprout novel synapses in more superficial layers of the dorsal horn, more commonly associated with nociceptive transmission (Lekan *et al.*, 1996), although more recently this work was repeated and found not to be the case (Hughes *et al.*,

2003). There may also be changes in neurotransmitters, receptors, ion channels and intracellular signalling in the CNS which may exist during chronic pain states.

Primary afferent nociceptors relay to projection neurons in the dorsal horn, which cross the spinal cord and ascend to supraspinal sites, and contribute to both affective and sensory aspects of pain (for review see Hunt and Mantyh, 2001). Projection neurons activate multiple higher centres, including the nucleus reticularis gigantocellularis, from here neurons project to the thalamus, and also activate the nucleus raphe magnus and periaquadual gray (PAG; Millan, 1999) of the midbrain.

Descending fibres from the PAG project also project to the nucleus raphe magnus and adjacent reticular formation. These neurons activate descending inhibitory neurons that are located in these regions and terminate in the dorsal horn of the spinal cord. Descending projections also arise from a number of brainstem sites, including the locus ceruleus. Several other sites within the limbic system receive projections from the spinal cord, such as the amygdaloid and septal nuclei (Fields *et al.,* 2006).

Some supraspinal structures which are involved in pain processing include the thalamus, cortical structures (including motor, premotor, parietal, frontal, occipital, insular and anterior cingulated regions), the PAG of the midbrain, which receives projections from a number of brain regions, including the amygdale, frontal and insular cortex and hypothalamus and acts in concert with the rostral ventromedial medulla (RVM) to provide descending pain modulation.

However, this thesis will focus on peripheral mechanisms of pain and inflammation; hence will not discuss central mechanisms further, although they clearly play important roles in all of the above processes.

1.7 HYPOTHESIS

The studies detailed in later chapters of this thesis were designed to test several hypothesis concering the mechanisms which lead to inflammation and pain of an inflamed joint. Firstly, it was expected that there would be significantly increased levels of known key inflammatory mediators in synovial cavities during the acute and chronic stages of adjuvant-induced arthritis, to which we could attribute the chronic pain, swelling and joint destruction seen during the model. These mediators, including TNFα, IL1β and IL6, are present in human arthritic joints and in other animal models of inflammatory joint disease. Once a suitable perfusion technique had been developed to enable sampling of joint fluid and therefore measurement of cytokine levels within the joint space, a 21-day time course of adjuvant-induced unilateral arthritis was carried out. Samples from ipsilateral and contralateral joints were taken and later assayed for cytokine levels. Blood samples were also taken to determine whether systemic levels of these mediators were elevated, which was expected based on previous evidence. A further hypothesis at this stage was that the levels of inflammatory mediators in the joints and in the blood would correlate to measues of joint swelling and hypersensitivity.

Chapters 6 and 7 were designed as a result of data generated in chapter 5. It was hypothesised from the expression levels of IL1β and IL6 in the knee joints during adjuvant-induced arthritis, and their stong correlation to the weight distribution readout during this time course, that they were directly involved in the induction of mechanical hypersensitivity both by direct actions on peripheral sensory nerves and via indirect actions on these primary afferents, probably via the release of secondary messengers. It was anticipated that intra-articular administration of either of these compounds would increase the electrical excitability of peripheral afferents, decreasing the mechanical threshold required to induce neural activity, increasing the rate of basal discharge and increasing the frequency of action potentials triggered to a suprathreshold stimulus. Furthermore, we hypothesised that IL1β and IL6, at the pharmacological doses administered, would initiate joint destruction,

including cartilage and potentially bone erosion, thus resulting in a chronic joint inflammation, including behavioural signs of swelling and hypersensitivity.

1.8 AIMS OF THE STUDIES

The primary aim of this thesis was to investigate the roles of specific pro-inflammatory cytokines in the development and maintenance of joint pain and inflammation. In order to achieve this two novel techniques were developed and validated with the view to using them alongside existing methods. A new behavioural tool to measure mechanical hypersensitivity directly across the knee joint which would add to current readouts was developed in collaboration with Dr Harry Brash, a medical physicist. This device was validated by determining whether it could detect changes in hypersensitivity of knee joints of rats and mice as a result of an inflammatory insult, and subsequently whether it was able to detect the reversal of hypersensitivity in animals treated with a range of gold-standard analgesic drugs. Secondly, a perfusion technique designed to improve the collection of synovial fluid from rat knee joints and to allow more accurate assessment of the inflammatory mediator and cell content of the joint cavity was developed. The perfusion technique was used to investigate the temporal expression patterns of inflammatory cytokines and cells in the knee joints of rats with adjuvant-induced arthritis, and the effect of the steroid prednisolone on these levels. Finally, the effects of IL1β or IL6 on

- acute and chronic joint swelling, hypersensitivity and joint destruction
- the release of other pro- and anti-inflammatory cytokines
- primary sensory afferent neural activity and sensitivity

were assessed using the two new techniques, as well as established methods including *in vivo* electrophysiological recording from primary afferent nerves innervating the rat knee joint, histological processing of normal and IL1β- or IL6-injected joints, the weight distribution readout and a measure of joint swelling.

Chapter 2. Materials and Methods

All experiments described in this thesis were performed in accordance with the regulations and guidelines of the Scientific Procedures Act (1986) under the Personal Licence number PIL 60/9007 and Project Licence, PPL 60/2750 until Aug 05, then PPL 60/3496. Animals used in these studies were housed at licensed animal facilities within the School of Biomedical and Clinical Laboratory Sciences and experiments were performed in licensed laboratories within the Division of Neuroscience at The University of Edinburgh. Joint perfusate samples were frozen and assayed for inflammatory mediator content at GlaxoSmithKline (Harlow, UK).

In these studies a total of 522 adult male Wistar rats and 24 adult male C57 Black-6 (C57BL/6) mice were used. All animals were purchased from Charles River (UK). Animals were kept on a 12-hour light/dark cycle and maintained on standard animal feed and had access to water *ad libitum* at all times.

2.1 MODELS OF EXPERIMENTAL ARHTRITIS

2.1.1 THE RAT FCA MODEL OF ARTHRITIS

Induction of unilateral arthritis

A localised chronic inflammation was induced in rats by intra-articular (i.art) injection of Freund's Complete Adjuvant (FCA, 1 mg ml^{-1}; heat killed *Mycobacterium tuberculosis* suspended in heavy liquid paraffin (HLP) oil with Monooleate adjuvant; Sigma, UK) into the left knee (stifle) joint under transient halothane anaesthesia (3% in oxygen). FCA (150 µl) was injected using a 26-gauge needle (Microlance, UK) inserted into the joint space through the patella tendon just below the patella. Once the animals recovered, they were housed in a cage (maximum four per cage) until they were used.

Induction of Bilateral Arthritis

A bilateral chronic inflammation was induced in rats by i.art injection of FCA (5 mg ml^{-1} heat killed *Mycobacterium tuberculosis* suspended in HLP oil with Monooleate adjuvant; MAFF, UK) into the left knee joint under transient halothane anaesthesia (3% in oxygen). FCA (100 µl; 500 µg) was injected using a 26-gauge needle (Microlance, UK) inserted into the joint space through the patella tendon just below the patella. Once the animals recovered, they were housed in a cage (maximum four per cage) until they were used.

2.1.2 CYTOKINE-INDUCED JOINT INFLAMMATION

A mild short-lived inflammation was induced in rats by i.art injection of recombinant rat IL1β or IL6 (Bioclone, USA) into the left knee joint under transient halothane anaesthesia (3% in oxygen). IL1β or IL6 (100 µl; 1, 3 or 10 µg) was injected using a 26-gauge needle (microlance, UK) inserted into the joint space through the patella tendon just below the patella. Once the animals recovered, they were housed in a cage (maximum four per cage) until they were used. Basal measurements of weight distribution, PAD LWT and knee joint diameter were made prior to administration of IL1β, IL6 or vehicle (t=0). The weight distribution between the hind limbs was assessed two, four and six hours post-IL1β or vehicle; PAD LWT measurements were made three, five and seven hours post-IL1β or vehicle. Measurements for body weight, joint diameter, weight distribution and PAD LWT were then made 24, 48, 72, 96, 144 and 168 hours post-IL1β or vehicle.

2.1.3 THE MURINE MODEL OF UNILATERAL ARTHRITIS

Male C57BL6 mice were injected with FCA (i.art; 20 µl; 200 µg). Briefly, injections were carried out during transient halothane anaesthesia (3% in oxygen) and a small incision was made over the left knee joint to allow visualisation of the patella tendon. FCA (20 µl) was injected under the patella tendon and directly into the joint space of the

knee joint using a 30-gauge needle mounted on a 50 µl Hamilton syringe. Once the animals recovered, they were housed in a cage (maximum eight per cage) until they were used.

2.2 ASSESSMENT OF ARTHRITIS

The single i.art injection of FCA produces a mild to moderate chronic inflammation that persists for up to 90 days (Wilson *et al.*, 2006). During this time, the general health of the animals remains good, as shown by their ability to eat, gain weight, groom and move around the cage as normal (Donaldson *et al.*, 1993; Gauldie *et al.*, 2004). Between the induction of arthritis and their use for joint perfusions or behavioural assessments, the animals' general health and mobility was observed by me and the Named Animal Care Worker. The level of inflammation and hypersensitivity associated with the experimental arthritis was determined prior to their use in further experiments; see Table 2.1 for experimental use times.

Experimental use	**Days post arthritis-induction**
Joint perfusions	FCA: 1 - 21 days
	IL1β, IL6, saline: 1 – 10 days
Histology	FCA or HLP: 14-21
	IL1β, IL6, saline: 1 – 10 days
Behavioural Studies	FCA or HLP: 1-28
	IL1β, IL6, saline: 1-7

Table 2.1 Experimental use times for joint perfusions, histological processing and behavioural studies of FCA-, HLP-, IL1β-, IL6-, or saline-injected rats.

2.2.1 SWELLING

The degree of joint inflammation was determined by measuring the diameter of the ipsilateral and contralateral joints using digital micro-callipers (Mitutoyo, Japan; see Figure 2.1). FCA (150 µg), IL1β or IL6 (1, 3 and 10 µg) injections resulted in a unilateral inflammation, restricted only to the ipsilateral joint, showing no spread to the contralateral side. However, studies designed to investigate the nature of the contralateral inflammation

using 500 μg FCA, resulted in both ipsilateral and contralateral swelling, the contralateral spread having a delayed onset of 10-14 days. Ipsilateral joint diameters were compared with contralateral measurement and the basal diameter, measured prior to intra-articular injections.

Figure 2.1 Photograph of the digital micro-callipers used to measure knee joint diameter of rats and mice.

2.2.2 WEIGHT DISTRIBUTION

The mechanical hypersensitivity as a result of i.art injections was determined by comparing the distribution of body weight between the two hind limbs using a dual channel weight averager (Linton Instruments; Clayton *et al.*, 1997). Briefly, the instrument consists of two force transducers capable of measuring the weight applied on each limb over a three second period. The rats were restrained for a short period in a clear Perspex box, which aligned the body so that each hind limb was placed on a transducer plate, with the fore limbs resting on a slope for support (see Figure 2.2). The force on each transducer was measured and given as a digital readout in grams for each limb. Using the contralateral joint as an internal control, the weight distribution could be expressed as a ratio (ipsilateral: contralateral) or as a percentage change from baseline. Usually, the animal distributed its weight equally between the two hind limbs prior to induction of joint inflammation, and then as arthritis develops the injected limb carried less weight than the contralateral joint.

Figure 2.2 Photograph of the incapacitance tester used to assess mechanical hypersensitivity of rats. A similar device with a smaller Perspex box and transducer plates is used for mouse measurements.

2.2.3 PRESSURE APPLICATION

A second method was also used to assess mechanical hypersensitivity of the joints, involving direct application of pressure to the joint. This novel apparatus, the Pressure Application Device (PAD) was developed and validated during my PhD and therefore makes a separate chapter (Chapter 4) in this thesis in which all the details are included.

2.3 IN VIVO RECORDING FROM AFFERENT NERVES

2.3.1 ANAESTHESIA AND SURGICAL PROCEDURES

Rats were initially anaesthetised with intra-peritoneal (i.p.) injection of pentobarbital sodium (60 mg ml^{-1}; 0.1ml kg^{-1}). Once fully anaesthetised, when no paw withdrawal reflexes occurred when the foot was pinched (areflexic), the animal was laid on its back and its core body temperature maintained at 37°C using an automated heating blanket (Harvard Apparatus Limited, UK) attached to a thermistor probe inserted into the rectum.

A small midline incision was made in the neck to expose the trachea, which was cannulated (cannula outer diameter (OD) 2.0 mm, Portex, UK) to allow the facilitation of breathing, or if required, artificial ventilation using a ventilator (Harvard Apparatus Limited UK). The right carotid artery was cannulated (cannula OD 0.75 mm) to allow the continual monitoring and recording of arterial blood pressure (BP) through a pressure transducer (Bell

and Howell, UK) attached to a Powerlab/ 8sp (ADInstruments, UK), connected to a personal computer running Chart 5.01 (ADInstruments, UK) software.

The medial aspect of the right hind limb was exposed by cutting the skin. The right femoral vein was cannulated (cannula OD 0.63 mm) to administer anaesthetic; deep anaesthesia and fluid levels were maintained throughout the experiment with an intravenous (i.v.) infusion of pentobarbital (375µl hr^{-1}; 7.5 mg hr^{-1}; 0.3-0.6 mg kg^{-1} min^{-1}) diluted 1:3 in saline (20 mg ml^{-1}). The infusion was adjusted regularly depending on reflexes, which were checked regularly by squeezing the intact paw for reflex withdrawal.

2.3.2 DISSECTION OF THE MEDIAL ARTICULAR NERVE

Extracellular recordings were performed on a portion of the MAN innervating the left knee joint (Freeman & Wyke, 1967; Gardner, 1944). The left hind limb was fixed to a support using plaster of Paris and a small incision was made on the medial aspect in order to expose the MAN where it branches from the saphenous nerve. The skin was secured with cotton stitches to a small brass ring to form a pouch which was filled with HLP oil to create an electrically isolated system (see Figure 2.3). The saphenous nerve was cut centrally to prevent interference from efferent neural activity. In addition, input from skin afferents was reduced by separating as much skin as possible from the knee joint. The MAN was dissected out from the surrounding tissue using fine forceps and placed over bipolar platinum/iridium (Pt/Ir) electrodes placed half way between the saphenous nerve and the joint capsule.

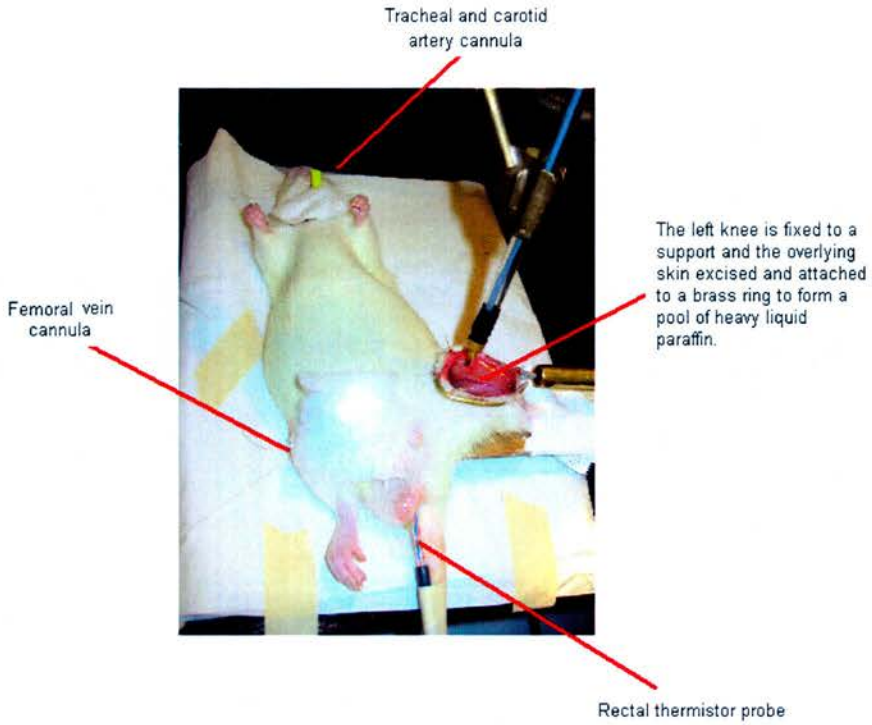


Figure 2.3 Photograph of the experimental set up for recording neural activity from sensory afferents in the MAN innervating the rat knee joint. The left knee joint was fixed to a support and the skin cut and tied to a small brass ring to form a pool, into which HLP was poured to produce an electrically isolated system. .

2.3.3 RECORDING FROM THE MAN

Afferent activity was recorded by laying the dissected MAN nerve bundle over the bipolar Pt/Ir electrodes. The raw nerve signal was passed through a pre-amplifier and an amplifier (Neurolog NL 103 and NL 105 respectively; signal amplified x 10 000). The signal was then displayed on an oscilloscope (Gould 1604), filtered (100 Hz low pass and 1000 Hz high pass; Neurolog, NL 115), and the voltage discriminated signal passed through a Micro 1401 interface (Cambridge Electronic Design (CED), UK) connected to a loud speaker and a personal computer (PC) running Spike 2 software (CED, UK; version 5.11). Electrical events were recorded online by setting both positive and negative trigger levels. These were saved as data files for off-line analysis. In addition, keyboard markers were written into the file whilst recording to identify drug injections or test points. Figure 2.4 illustrates the experimental set-up. Units were characterised only to mechanical stimulation,

in order to reduce the number of stimuli applied, which may affect the responsiveness of the nerves.

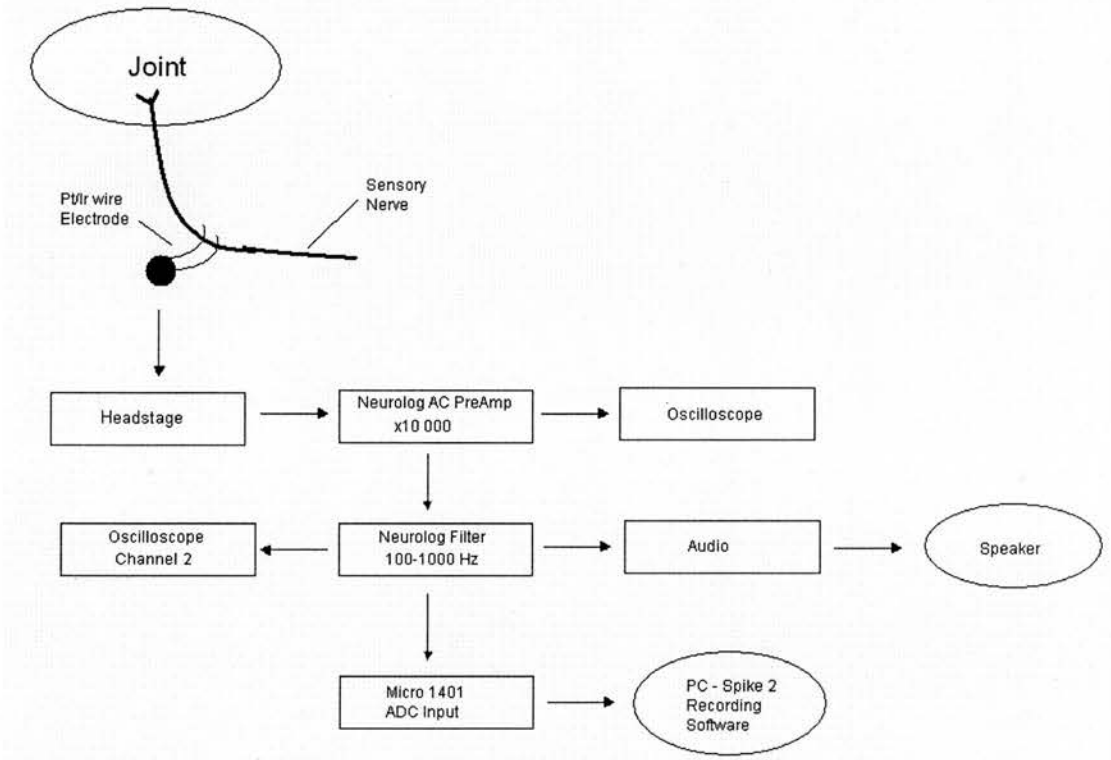


Figure 2.4 A schematic diagram of signal processing during neural recording illustrating the equipment used to record, store and analyse the neural activity from the sensory afferents innervating the rat knee joint.

2.3.4 DRUG ADMINISTRATION

Drugs were administered via an i.art injection into the left knee joint. Recombinant rat IL1β (0.1 μg in 100 μl) or IL6 (0.1 μg in 100 μl) were administered i.art into the left knee joint two hours after the nerve recording began, to allow the nerve to stabilise following the preparatory surgery, and to allow two control tests to be carried out. The electrode was lowered, reducing the tension of the nerve, in order to prevent damage during movement of the joint during the injection, and then raised immediately after. A volume of 100 μl was administered into the knee joint using a 25-gauge needle. The drugs used in this study, including molecular mass and suppliers are given in Appendix 1.

2.3.5 MECHANICAL STIMULATION

The threshold to mechanical stimulation was determined using a range of six calibrated von Frey hairs, 0.96, 2.35, 4.64, 7.37, 12.5 and 20.9 g (Stoelting, Scientific Marketing Associates, UK); once the nerve responded to a fibre, no higher forces were applied. Two tests were carried out 30 minutes apart before (control; -60 and -30 minutes) administration of IL1β, IL6 or saline and then 15, 30, 60, 90, 120, 150, 180, 210 and 240 minutes after.

In a separate series of experiments, the discharge evoked by 0.98, 12.5 or 20.9 g von Frey hairs was measured at regular intervals before and after i.art administration of IL1β, IL6 or saline. The receptive field of the nerve was located by probing with a plastic tip 1 mm in diameter, and a specific location for repeated stimuli was marked to ensure accurate repeated applications of the fibres. The von Frey filament was applied for a five second period and then removed. Two tests were carried out 30 minutes apart before (control; -60 and -30 minutes) administration of IL1β, IL6 or saline and then 15, 30, 60, 90, 120, 150, 180, 210 and 240 minutes after.

2.3.6 BASAL NEURAL ACTIVITY RECORDED FROM THE NERVE

Changes in basal discharge frequency were studied following administration of IL1β (0.1 μg in 100 μl ;n=8), IL6 (0.1 μg in 100 μl ;n=8) or vehicle (100 μ n=5); 300 second time periods were used to assess the firing frequency at two time points prior to IL1β, IL6 or vehicle administration (t= -60 and -30 minutes) and then again 15, 30, 60, 90, 120, 150, 180, 210 and 240 minutes after IL1β, IL6 or vehicle administration.

2.3.6 DATA SORTING

Once the experiment was recorded on the PC, the neural data was analysed using Spike 2 software (see Figure 2.5a). The recorded waveform was scanned for action potentials that exceeded the noise band and then rescanned and waveform templates used to

represent the individual spikes that make up the responses. Once this was achieved, the response of each unit was displayed on individual channels (see Figure 2.5b). Data was generally displayed in histogram format, showing the total number of impulses in one second, providing data on the absolute discharge (impulses) and frequency (impulses s^{-1}).

2.3.7 DATA ANALYSIS

Responses to mechanical stimulations were determined by comparing the action potential discharge frequency or the absolute number of action potentials recorded during the test period with that of the basal discharge recorded prior to mechanical stimulation. Data are expressed as either the change in mean action potential frequency ($\Delta\bar{\chi}$; impulses s^{-1}; Equation 1) or the change in the absolute number of action potentials evoked over the duration of the response ($\Delta\sum x$; impulses; Equation 2) between that of the test period and that of the control period immediately prior to the test. If no clear response was observed the afferent activity present was measured for 15 s after the injection.

$$\Delta\bar{\chi} = \bar{\chi}_{test} - \bar{\chi}_{control} \qquad \text{Equation 1}$$

$$\Delta\sum\chi = \sum\chi_{test} - \sum\chi_{control} \qquad \text{Equation 2}$$

Definitions:

$\sum\chi$: The total number of action potentials counted in the control (15 s) or test periods, $\sum\chi_{control}$ or $\sum\chi_{test}$ respectively (expressed as impulses).

$\bar{\chi}$: The frequency of action potential discharge in the control (15 s) or test periods (expressed as impulses s^{-1}).

Therefore,

$$\bar{\chi}_{control} = \sum\chi_{control} / t_{control}$$

$$\bar{\chi}_{test} = \sum\chi_{test} / t_{test}$$

Where changes in spontaneous action potential discharge were studied following i.art administration of compounds, 300 second time periods were used to assess the discharge

frequency at two time points prior to substance administration (-60 and -30 minutes; controls) and then again at 15, 30, 60, 90, 120, 150, 180, 210 and 240 minutes after.

The mechanical threshold to von Frey filaments was used to assess the affect of i.art IL1β, IL6 or saline on primary afferent sensitivities. Two control measurements were made 30 minutes apart before injection of compounds into the synovial cavity (-60 and -30 minutes) and then again at 15, 30, 60, 90, 120, 150, 180, 210 and 240 minutes after.

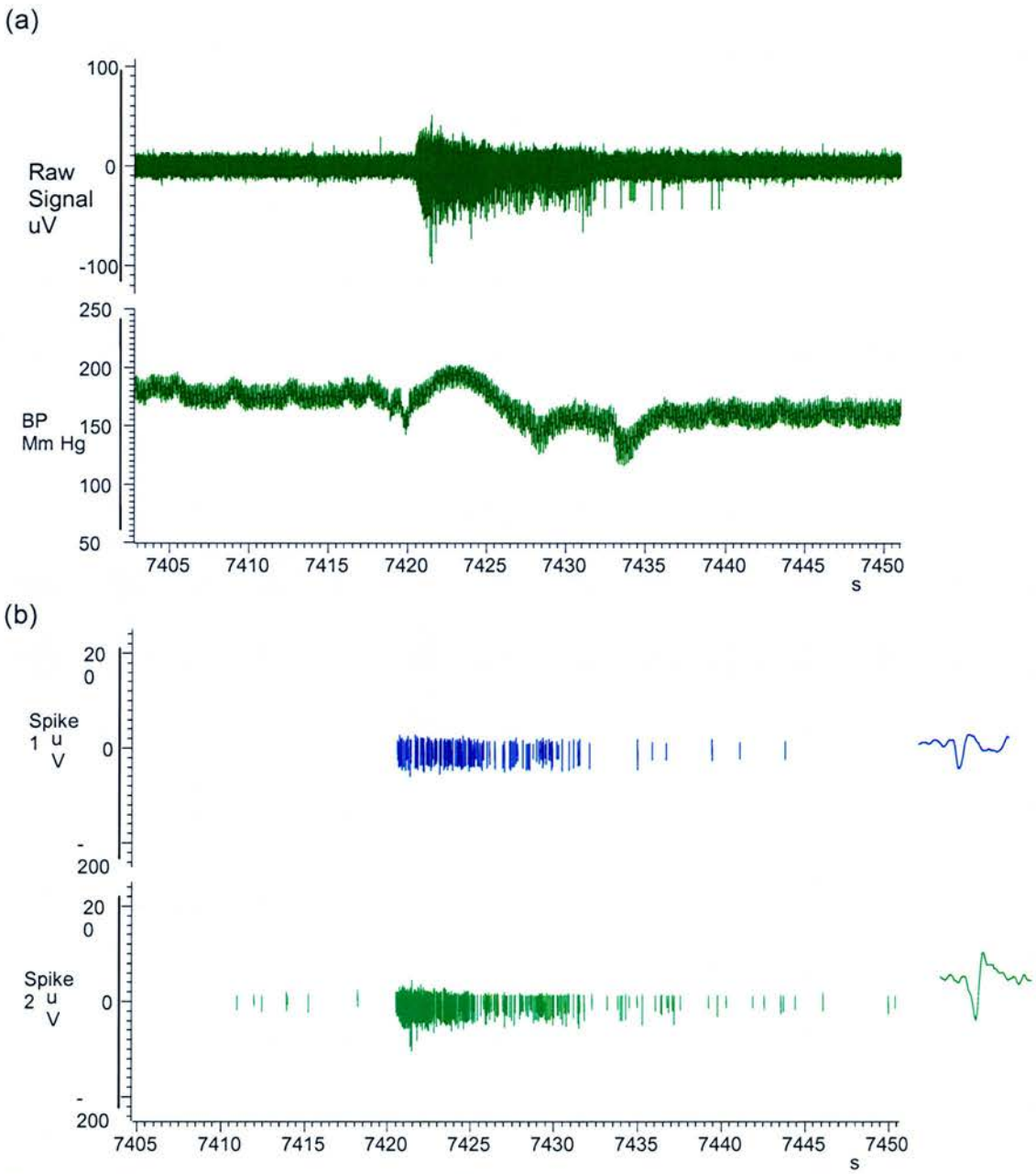


Figure 2.5 Spike traces showing (a) the raw filtered neural signal (top) and the BP trace (bottom) for an i.a. injection of 1μg Capsaicin and (b) the individual fibre patterns of two units in the bundle during the same injection and the spike shapes of the 2 units in the recording.

2.4 RAT KNEE JOINT PERFUSION

As the development of this novel technique comprised a significant portion of the overall project, it is covered in a separate chapter in this thesis. Full details of the method are provided in chapter 4.

2.4.1 THE PERFUSION NEEDLES

A needle perfusion system was constructed by binding a 25- and a 23-gauge needle (Microlance, UK) together using epoxy putty, with the bevels of the needles positioned on the outside edges facing away from one another (see Figure 2.6). The tips of the needles were set 1-1.5 mm apart. The needles were connected to a Watson-Marlow roller pump via silicone rubber perfusion tubing (internal diameter 1 mm, external diameter 4.2 mm, Watson Marlow, UK).

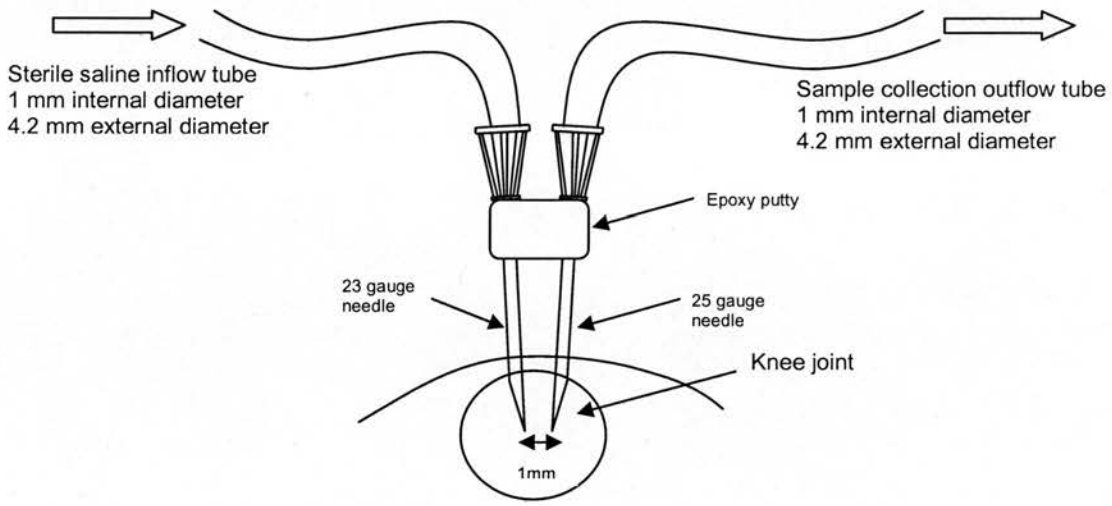


Figure 2.6 The perfusion needles and the perfusion system managing inflow and outflow from the knee joint space. A Watson-Marlow pump controlled the rate of saline infusion and sample extraction (100 $\mu l\ min^{-1}$) from the joint.

2.4.2 PERFUSION OF THE KNEE JOINT

Rats were anaesthetised with i.p. urethane (ethyl carbamate; 0.6 ml $100g^{-1}$ body weight; 25% w v^{-1} solution; single i.p. injection) or sodium pentobarbital (1 ml kg^{-1} body weight; 60 mg ml^{-1} solution; single i.p. injection maintained with i.v. 375 $\mu l\ hr^{-1}$ 20 mg ml^{-1}

solution). Once fully anaesthetised the animal was placed on its back on an automated heating blanket (Harvard Apparatus Limited, UK) and its core body temperature maintained at 37°C via a thermistor probe positioned in the rectum.

The limbs of the rat were flexed over a 20 ml glass vial, with the patella facing directly upwards and were held in place with tape. The perfusion needles were inserted to a depth of approximately 3 mm into the joint and held in place with the aid of a clamp attached to the table. The 23-gauge needle was connected to the Watson-Marlow roller pump via silicone rubber perfusion tubing and sterile saline was infused at a constant rate of 100 µl min^{-1}. After infusion of 100 µl of sterile saline, the outflow tubing was connected to the 25-gauge needle, to minimise pressure build-up within the joint space. Fluid was infused and withdrawn at a constant rate until a 250 µl sample was collected in a 1.5 ml centrifuge tube. Samples were immediately frozen and stored at -20°C. Approximately 75% of joint perfusions resulted in successful sample collection. Problems that resulted in collection failure included inconsistent outflow of solution from the joint space or incorrect needle placement. Samples that were contaminated with blood were not used.

2.4.3 CYTOKINE ASSAY OF JOINT SAMPLES

ELISA Assay

PGE_2, TNFα and IL1β content of samples were measured using commercially available ELISA kits (PGE_2: Amersham Biosciences, UK; rat IL1β and TNFα: BioSource International, Camarillo, USA), according to the manufacturers' protocol. The ELISA assay for PGE_2 was carried out by Ms Penny Staton at GlaxoSmithKline, Harlow, UK.

Briefly, 50 or 100 µl aliquots of sample were pipetted into the wells of a microtiter plate pre-coated with an antibody specific for rat IL-1β, TNFα or PGE_2 and incubated for between 1 and 3 h at room temperature. After washing, a different biotinylated anti-rat IL-1β, TNFα or PGE_2 antibody was added and incubated at ambient temperature for 1 h. Streptavidin-peroxidase (HRP) was added and incubated for 30 – 45 min. After a third

incubation and washing to remove all unbound enzyme, colour was developed by addition of stabilized chromogen (tetramethylbenzidine), a stop solution added after 30 minutes and the intensity of the coloured product quantified by a 96-well spectrophotometer at 450 nm (Thermo Labsystems Multiskan Ascent plate reader running Ascent software Version 2.6). The minimum detection limit of the assay was 3, 4 and 50 pg ml^{-1} for IL1β, TNFα and PGE_2 respectively.

ELISA Data Analysis

Standard curves were plotted using the aliquoted serial dilutions of a positive control solution for calibration. Unknown sample cytokine concentrations were calculated from the curve.

Luminex Bead Array Assay

Perfusates were analysed for multiple analytes using a multi-cytokine bead array detection system. Rat IL1α, IL1β, IL2, IL4, IL6, IL10, IFNγ, GM-CSF and TNFα were measured using a Bio-Rad rat 9-plex kit, according to the manufacturer's instructions (Biorad, USA). A monocolonal antibody directed against the desired analyte is covalently coupled to dyed 5.5 μm polystyrene beads ($2.5x10^6$ beads ml^{-1} $cytokine^{-1}$). The conjugated beads were exposed to 50 μl of sample or standard solutions containing a known amount of cytokine, in a 96-well filter plate and incubated overnight at 4°C, protected from light. After a series of washes with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and vacuum filtration (Millipore, USA) to remove unbound protein, a biotinylated detection antibody specific for a different epitope on the analyte was added to the reaction, After incubation, the unbound antibody was removed; the reaction mixture was detected by the addition of streptavidin-phycoerythrin (streptavidin-PE; Europa Bioproducts, UK), which binds to the biotinylated detection antibodies. Following three washes and vacuum filtration, the beads were re-suspended in 200 μl 5% BSA in PBS; the plate was stored at 4°C in the

dark until analysis, up to a maximum of 24 hours later. The reaction mixture was read using a Luminex Data Collector in a Luminex xMAP 100 (Luminex, USA).

Luminex Data analysis

Excel data files were generated by the Luminex containing individual bead numbers and the associated median fluorescence intensities. Standard curves were plotted to calculate the relative amount of each cytokine in samples, using the aliquoted serial dilutions of a positive control solution for calibration. Cytokine concentrations of unknown samples were calculated from the curve.

2.4.4 TOTAL CELL COUNTS OF PERFUSION SAMPLES

Total inflammatory cell counts of perfusion samples were determined using 10 µl of sample collected from rat knee joints as described above but with 3.15% sodium citrate in the sterile saline infusion solution. Samples were viewed in a haemocytometer using a light microscope. Initially undiluted samples were examined, but due to the red blood cell content of some samples, it was necessary to dilute the samples with saline with added Zappoglobin (1-2 drops per 20 ml saline; a red blood cell lysing agent). All cells remaining could be identified as white blood cells (inflammatory), and were counted, and multiplied up by the dilution factor and 10, 000 (as a 10 µl sample used) to calculate the total number of inflammatory cells in 1 ml of joint perfusion sample. Inflammatory cell count analysis was carried out at The Queens Medical Research Institute, Little France, Edinburgh in collaboration with the laboratory of Prof. Adriano Rossi.

2.4.5 BLOOD SAMPLE COLLECTION

Blood samples were taken from the tail vein after perfusion of the knee joints was complete. These were collected in EDTA-coated capillary tubes (Sarstedt Microvette, UK) and centrifuged (Eppendorf Centifuge, Model 5414) at 12000 revolutions per minute (rpm)

for ten minutes. The plasma was removed and placed in a 1 ml centrifuge tube and frozen at -20° C. Blood samples were later assayed for multiple cytokines using the Luminex assay.

2.5 HISTOLOGICAL PROCESSING

The left knee joints of rats were injected with IL1β (1 μg; n=12) or IL6 (1 μg; n=12) under transient halothane anaesthesia (3% in oxygen). On days 1, 2, 4 and 7 post-IL1β or -IL6 three animals were killed and the hind limbs surgically removed and fixed in formalin. Animals were killed by rising carbon dioxide concentrations in a chamber, and both the left and right knee joints removed and fixed in 10% formal saline. All tissues were then processed (post-fixed, decalcified and paraffin embedded), sectioned (3 μm sections) and mounted on glass slides by Professor Donald Salter (Department of Pathology, University of Edinburgh). Paraffin-embedded sections of rat knee joints were stained using haematoxylin and eosin (H&E), and covered with glass coverslips using Pertex mountant. The sections were subsequently examined using light microscopy by Prof. Donald Salter, at The Department of Pathology, Royal Infirmary of Edinburgh

2.6 STATISTICAL ANALYSIS

Data was collected and analysed using Microsoft Excel, GraphPad Prism and GraphPad Instat software. Data is expressed as mean ± standard error of the mean (SEM) where appropriate. Unpaired t-tests were used to analyse the differences between the means of two normally distributed groups. When the sample size for each group was small, or data was not normally distributed the non-parametric Mann-Whitney U-test was performed. For paired data the Student's paired t-test (parametric) or Wilcoxon U-test (non-parametric) tests were used. To determine whether statistically significant differences existed between group mean values a one-way analysis of variance (ANOVA) was done and a post-hoc test (Dunns multiple comparison) performed if the result was deemed to be significant. The means of two or more groups of non-parametric data were analysed with a Kruskal-Wallis test and a

post-hoc analysis done using Dunn's multiple comparison. Correlations between normally distributed groups were determined using Linear (Pearson) correlation and non-parametric data were compared using a Spearman Rank Correlation. To compare the relative proportions in a population between two groups the Fisher's Exact test was used. Where the effects of drug-treatment were assessed, area under the curve (AUC) values were calculated during the dosing period and compared with that of vehicle-treated groups. AUC values were calculated for individual animals on each day during the dosing period. The baseline was taken as the measurement on the day prior to drug treatment. AUC values were compared with vehicle using a Kruskal Wallis non-parametric ANOVA. In all cases the null hypothesis was rejected at the 0.05 level. Therefore $P \leq 0.05$ was considered statistically significant and where possible the calculated P value was quoted to show the proximity to the 0.05 limit.

Chapter 3 – Development of an automated pressure withdrawal threshold readout (PAD) to assess mechanical hypersensitivity of rat and mouse knee joints

3.1 INTRODUCTION

One of the main symptoms of arthritis is joint pain (Anderson *et al.*, 1994; Heiberg & Kvien, 2002; Kazis *et al.*, 1983; McKenna & Wright, 1985); quantification of this is useful in determining the mechanisms of pain and evaluating the effects of anti-arthritic, analgesic or anti-inflammatory drugs. Pain is not a single sensory experience, and thus a wide variety of models have been developed to represent different types of pain. Although acute thermal and mechanical assays exhibit adequate validity for many analgesics (Taber, 1973), acute pain is not a clinically relevant situation, particularly for RA patients. Thus, a number of animal models of "chronic" joint pain have been developed, including adjuvant-induced arthritis, as discussed in Section 1.5.1.

Measurements of spontaneous pain as well as hypersensitivity states, such as hyperalgesia or allodynia, are assessed in these models. A range of behavioural tests are used to determine nociceptive thresholds to thermal, mechanical or electrical stimuli (for review see Le Bars *et al.*, 2001). Spontaneous measures of pain in laboratory animals include assessment of weight-bearing by the affected foot (Clayton *et al.*, 1997; Min *et al.*, 2001; Schott *et al.*, 1994), locomotor activity, foot position and gait analysis, either subjectively or with the use of a computer (Clarke *et al.*, 1997; Coderre & Wall, 1988; Gegout-Pottie *et al.*, 1999; Otsuki *et al.*, 1986; Wang *et al.*, 2000) and paw elevation time of a rat walking on a rotating cylinder (Tonussi & Ferreira, 1992).

The incapacitance tester (Clayton *et al.*, 1997) consists of two force transducers on which the animals' hind limbs are positioned; the device is able to provide an objective, non-evoked assessment of pain by measuring the average weight placed independently on each hind limb. Naïve rodents distribute their weight evenly across both hind limbs under normal conditions (Kobayashi *et al.*, 2003). Following induction of joint inflammation, animals redistribute their weight in order to place less weight on the affected joint, as a guarding

mechanism to protect the limb and prevent further injury during tissue repair. Results based on the incapacitance tester were found to be less variable and more graded that those of a subjective grading of animal stance (Schott *et al.*, 1994).

Mechanical stimulation of the paw using von Frey filaments (Chaplan *et al.*, 1994) or the Randall-Selitto apparatus (analgesymeter; Randall & Selitto, 1957) is commonly used to assess inflammatory pain. However, although these methods appear to measure arthritic pain, there are problems associated with their use as an index of pain in a particular joint, such as the knee. For example, testing sensitivity of the paw to assess pain in the knee joint is indirect.

Recently, home-made calibrated forceps which apply a gradually increasing pressure to the paw until the animal withdraws the paw or vocalises have been used to assess pain (Gauriau & Bernard, 2004; Yu *et al.*, 2002). These devices consist of two strain gauges attached to the inner arms of large forceps. The strain gauges are electrical resistors; the resistance varies linearly according to the strain applied, and the withdrawal threshold is automatically recorded when the animal withdraws the paw. One study has used a similar technique on the knee joint (Neugebauer & Li, 2002), but used vocalisation as the test endpoint rather than limb withdrawal, which meant that supraspinal responses, rather than basic spinal nociceptive reflexes were being investigated. Application of a compression or squeeze force to the joint is more relevant than punctate mechanical stimulation of the skin overlying the joint with von Frey filaments, which is more likely to stimulate skin nociceptors, rather than nociceptors in the joint.

Animal models are designed to mimic human disease, so it is crucial that they represent human arthritic pain, and that the assessments of this pain used in the laboratory accurately reflect those used clinically. Pain is "subjective" if reported from patient self-report questionnaires or "objective" if data is interpreted from laboratory, imaging and physical examination (Sokka, 2003). Patient self-report questionnaires have been developed to facilitate qualitative and quantitative assessment of pain status and functionality of arthritic joints and

have proven valuable in gaining new understandings of mechanisms and control of pain (Anderson, 2001; Bellamy, 1989; Bellamy *et al.*, 1988; Fries *et al.*, 1980; Hunt *et al.*, 1985; Huskisson, 1974; Meenan *et al.*, 1980; Melzack, 1975; Ware & Sherbourne, 1992). Furthermore, quantitative pain measures from patient questionnaires provide information to recognize if patients improve or worsen over time. For example, the self-report health assessment questionnaire contains a scale of 20 activities of daily living in eight categories, designed to assess functional disability with four response options for the patient:

- "without difficulty" (0)
- "with some difficulty" (1)
- "with much difficulty" (2)
- "unable to do" (3)

Efforts to assess pain in patients through objective external measures have not been successful in providing reliable quantitative data (Sokka, 2003). Furthermore, the most effective predictor of mortality in patients with RA is found in a patient questionnaire, rather than in data from physical examination, laboratory tests or radiography (Pincus *et al.*, 1994; Pincus *et al.*, 1984). However, with the inability to communicate verbally with experimental animals, physical behavioural measures of pain are the only way to assess experimental hypersensitivity.

Three functional measures used for assessing RA patients in the clinic include "grip strength" (Lee *et al.*, 1974), the "button test" (Clawson *et al.*, 1971) and "walk time" (Deodhar *et al.*, 1973). The "grip test" uses a BP cuff inflated to 20 mm of mercury. The patient is asked to squeeze as hard as possible, triplicate measurements are taken for each hand, and the average value used as the grip strength value. To assess "walking time" patients are asked to walk at a "normal" pace for a set distance; the test is timed using a stopwatch. The "button test" uses a standard commercially available board; patients are asked to button and unbutton five shirt

buttons attached to the board as quickly as possible, the process is timed. When performed according to a standard protocol (Pincus & Callahan, 1992) these methods are among the most reproducible measures in clinical rheumatology (Pincus *et al.*, 1994; Pincus *et al.*, 1987).

A dolorimeter is an instrument which applies progressive pressure to the joints of patients who are asked to report when they first experience pain and when the pain becomes unbearable. Dolorimeters have been used for many years to measure the pain threshold, which is more accurately assessed than pain itself. The dolorimeter has proven to be of great value in research concerning pain thresholds and behaviours (Langley *et al.*, 1983). Furthermore, the dolorimeter was found to be more sensitive than a modified Ritchie index in measuring the degree of joint tenderness and equally as sensitive in detecting tender joints. The Ritchie articular index is generally accepted as a standard subjective method for measuring joint tenderness in RA (Ritchie *et al.*, 1968) and is widely used in evaluating the response to anti-rheumatic therapy during clinical trials. The Ritchie articular index allocates one of four grades of tenderness to patients' joints:

- "Not tender" (0)
- "Tender" (1)
- "Tender and winced" (2)
- "Tender, winced and withdrew" (3).

The dolorimeter was able to detect changes in joint tenderness in a drug withdrawal study, whereas the Ritchie index was not (Langley *et al.*, 1983).

In this chapter the development and use in our laboratory of a novel behavioural readout, capable of being used in both mice (Strickland *et al.*, 2005) and rats is described. The pressure application device (PAD) uses similar principles to the clinical pressure dolorimeter and was designed to apply a gradually increasing squeeze directly across a knee joint until the rodent

shows signs of pain or discomfort, as evidenced by withdrawal of the limb, or rarely vocalisation. At this "end point" a quantitative value of the maximum force applied prior to limb withdrawal is recorded, and this value provided an objective measure of the withdrawal threshold. The aim of the study was to evaluate accuracy, sensitivity, and reliability of the novel device designed to measure knee joint hypersensitivity in rats and mice with experimentally-induced arthritis, in comparison with untreated joints. PAD was also used to determine whether it could detect reversal of hyperalgesia by analgesics known to be clinically effective in relieving the pain of arthritis.

A dose-response study using prednisolone (Pyne *et al.*, 2004; Song & Buttgereit, 2006), and other studies using morphine (Caldwell *et al.*, 2002; Dickenson & Kieffer, 2006) and celecoxib (Schnitzer *et al.*, 2005; Hawkey, 2001), were performed to investigate the reproducibility and sensitivity of PAD in rats, whilst comparing the device to the already established weight distribution readout. A smaller study was carried out in mice, in which only the effects of prednisolone were investigated.

3.2 MATERIALS AND METHODS

3.2.1 PAD- KNEE JOINT WITHDRAWAL THRESHOLD MEASUREMENT

PAD was designed and constructed by Dr Harry Brash, a medical physicist at The University of Edinburgh, working in collaboration with our laboratory on this project. PAD consists of a force transducer mounted on a unit fitted to the experimenter's thumb (see Figure 3.1). The thumb unit is connected to a recording base unit containing the control panel and digital readout display. The rat apparatus has a force transducer with a range of 0-1500 g (Honeywell, FSG-15N1A, Farnell, UK) and the diameter of the circular contact area is 8 mm; the area of the surface is therefore 50.3 mm^2. Due to the difference in size between mice and rats, a second force transducer was developed for use in murine studies, which had a force range

of 0-500 g (Honeywell, FSL05N2C, Farnell, UK) and a 5 mm diameter contact area, hence a 19.6 mm^2 area. All measurements were made in Edinburgh, UK, therefore all values are comparable, as gravity was consistent.

Animals were lightly, but securely held (rats) or scruffed (mice) by Mrs Susan Bond for the rat studies or by Mr Iain Strickland during the mouse study. The operator (myself) placed the thumb unit on one side of the animal's knee joint and the forefinger on the other. A gradually increasing force was applied across the joint by squeezing, and the force in grams applied was displayed on the digital screen. The test endpoint was when the animal reflexly withdrew its limb or on the rare occasion when the animal vocalised prior to limb withdrawal. The peak gram force (gf) applied immediately prior to limb withdrawal was recorded by the base unit, and was designated the limb withdrawal threshold (LWT). Three measurements were made at one minute intervals and the mean LWT was calculated. Both ipsilateral (left) and contralateral (right) limb measurements were made.

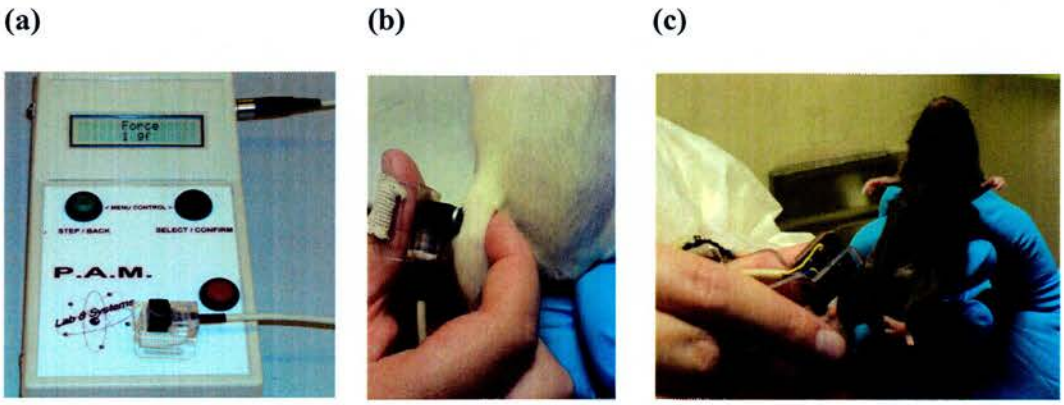


Figure 3.1 Presentation of the pressure application device (PAD). (a) The portable control unit with digital display and thumb attachment and PAD in use measuring the LWT of (b) rat and (c) mouse.

3.2.2 STUDY DESIGN AND DRUG TREATMENT

A pilot study was performed in rats to determine whether PAD could detect hypersensitivity following i.art (left – ipsilateral) FCA in comparison with naïve animals. The

study also compared values for PAD with those obtained in the same animals using the incapacitance tester. Baseline values for body weight, weight distribution, PAD LWT and knee joint diameters were measured prior to induction of the joint inflammation (on day 0), and were repeated 2-3 times weekly until day 28.

Subsequently, a drug-treatment study was carried out to investigate whether PAD was able to detect the reversal of joint hypersensitivity and how this differed in rats treated with three different types of drug, an opioid, a steroid and an NSAID. Baseline values for body weight, weight distribution, PAD LWT and knee joint diameters were measured, prior to induction of joint inflammation (left – ipsilateral joint). Measurements were made two or three times a week until 10 days post-FCA, when animals were randomly assigned into treatment groups. Drugs were administered subcutaneously (s.c.) in a volume of 2 ml kg^{-1} between days 14 and 18; drugs were administered blindly by Mrs Susan Bond. Prednisolone (1, 3 or 10 mg kg^{-1}; Rioja *et al.*, 2004) was injected once per day; whereas morphine (3 mg kg^{-1}; Wilson *et al.,* 2006), celecoxib (15 mg kg^{-1}; Pinheiro & Calixto, 2002) and vehicle (ethanol 5%, Polyethylene Glycol (PEG) 45% and distilled water 50%) were given twice a day. One hour after dosing blind behavioural assessments were made by myself following a strict protocol in order to give animals a rest period between the weight distribution and the PAD measurements. Briefly, animals were weighed and their weight distribution assessed by taking three consecutive readings before being returned to their cage. Five minutes later, animals had the sensitivity of the ipsilateral and contralateral knee joints assessed using PAD, with three readings taken. Finally the knee joint diameters were measured. Further behavioural assessments were carried out once the dosing period was complete, on days 21, 24 and 28.

A smaller study was carried out in mice as the focus of our laboratory is mainly on rat models of inflammation. However, PAD was developed for mice, due to the increasing use of genetically modified mice in science. For the murine study only prednisolone (1 mg kg^{-1}) or

vehicle was used. Drugs were administered blind (s.c.) by Mr Iain Strickland once a day in a volume of 3 ml kg^{-1}. All groups were assessed for behavioural changes daily during the dosing period one hour after dosing on days 13-17 post-FCA (left – ipsilateral joint). Further behavioural assessments were carried out on day 21, once the dosing ended.

3.3 RESULTS

In all the studies performed, i.art injection of FCA had no adverse effects on the general health of the animals. They continued to feed and gain weight normally throughout the studies (see Figure 3.2) and there was no significant difference in body weight between any of the FCA-injected and sham animals at any time point in rats or mice, or between vehicle- and drug-treated animals ($P>0.05$, Kruskal-Wallis).

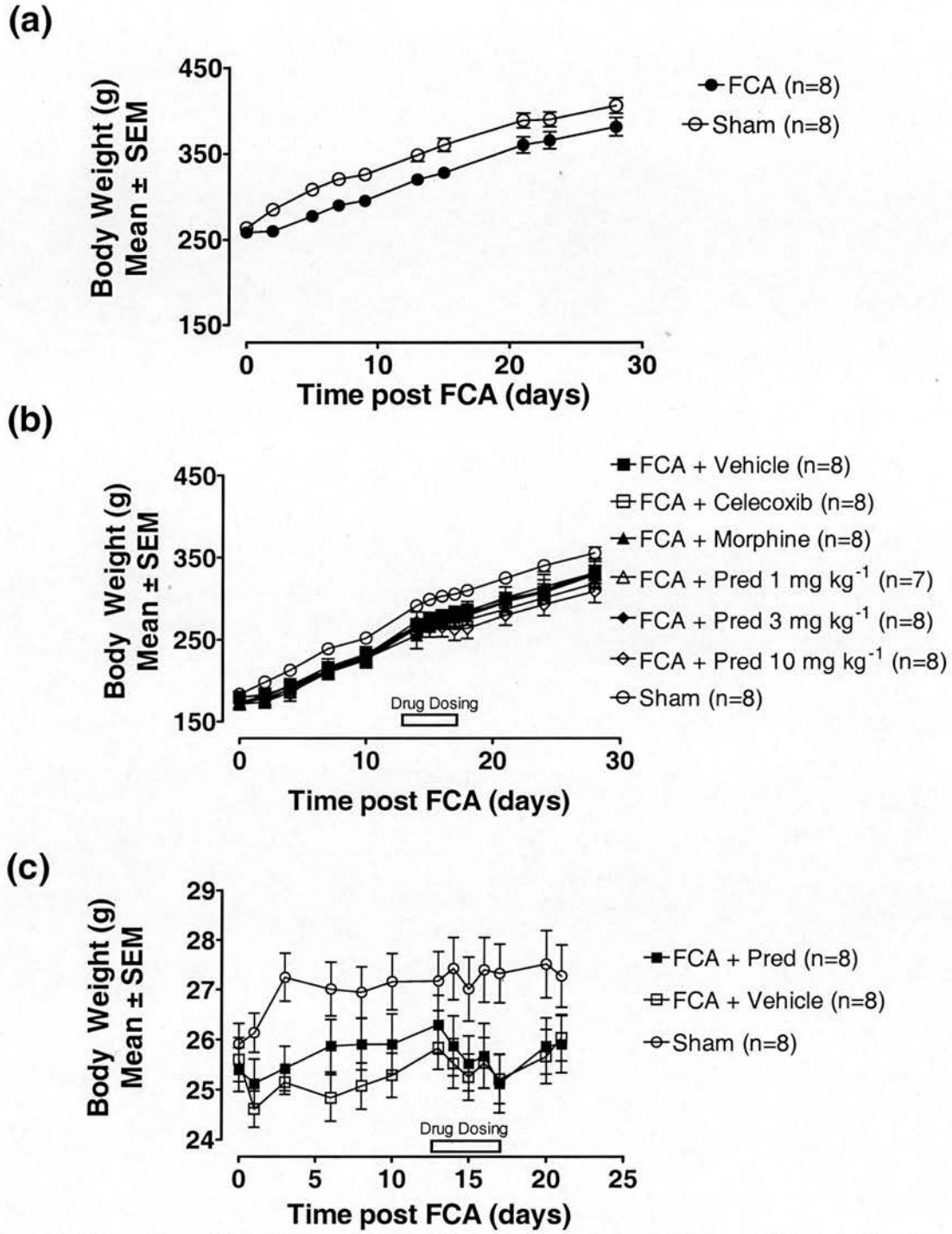


Figure 3.2 The effect of i.art FCA on body weights of animals during (a) the rat pilot study, (b) the rat prednisolone, morphine and celecoxib drug study and (c) the murine study. Although there were no significant differences in body weight between FCA-injected and sham animals ($P>0.05$, Kruskal Wallis), there was a trend for FCA-injected animals to have slightly lower body weights following FCA injection.

3.3.1 FCA-INDUCED HYPERSENSITIVITY IN RATS - PILOT STUDY

Pressure application device - PAD

Prior to induction of joint inflammation (on day 0) the average ipsilateral LWT was 710 ± 41 gf (n=16) and the average contralateral LWT was 790 ± 39 gf (n=16), these were not significantly different from each other ($P>0.05$, paired t-test). There was also no significant difference between the ipsilateral LWTs of the two study groups at this time point ($P>0.05$, Mann Whitney).

On day 1, following i.art FCA, the average ipsilateral LWT, decreased by 57% to 316 ± 45 gf (n=8), this was significantly lower than both the day 0 value ($P<0.05$, Wilcoxon) and the sham group at the same time point ($P<0.05$, Mann Witney; see Figure 3.3). Despite being untreated the ipsilateral LWT of sham animals increased significantly by 57% to 1028 ± 48 gf (n=8; $P<0.05$, Wilcoxon) on day 1 compared with day 0 values. Similar increases were seen in contralateral joints of both groups over the first 24 hours of the study (see Figure 3.3). Subsequently, this measurement remained relatively steady at approximately 1000 gf for the duration of the study in all contralateral joints, and ipsilateral joints of sham animals. The average ipsilateral LWT of FCA-injected rats was significantly lower than sham and contralateral joints on each test day over the full 28 day time course studied ($P<0.05$, two-way ANOVA).

Incapacitance Tester – Weight Distribution

On day 0, the average weight placed on ipsilateral and contralateral hind limbs in rats was 96 ± 3 g and 98 ± 3 g respectively, resulting in a ratio of 0.98 ± 0.03 (n=16). There was no significant difference between the two groups ($P>0.05$, Mann Whitney). The ratio of weight distribution between the hind limbs for FCA injected rats decreased by 70% (to 0.29 ± 0.05; n=8; see Figure 3.3) on day 1, which corresponds to 40 ± 4 g and 140 ± 5 g placed on ipsilateral

and contralateral limbs respectively. The ratio was significantly less than that in sham animals ($P<0.05$, Mann Whitney), which had a ratio of 1.05 ± 0.02 (ipsilateral = 116 ± 4 g and contralateral = 110 ± 4 g; n=8; see Figure 3.3) at this time point. The ratio remained significantly reduced in FCA-injected animals compared with the sham group on each test day up until and including day 28 ($P>0.05$, two-way ANOVA). There were no significant changes observed in the sham group at any time point in this behavioural readout ($P>0.05$, Kruskal Wallis).

Joint Swelling

Basal measurements of joint diameter were 9.92 ± 0.08 mm and 9.85 ± 0.12 mm for ipsilateral and contralateral knee joints respectively (n=16). These values did not differ significantly from one another ($P>0.05$, Wilcoxon), with no significant differences observed between the two groups ($P>0.05$, Mann Whitney). On day 1, the average ipsilateral knee joint diameter of FCA-injected rats increased to 13.11 ± 0.06 mm (n=8), compared with 10.12 ± 0.1 (n=8) in sham animals; this was a statistically significant increase ($P<0.05$, Mann Whitney; see Figure 3.3). The swelling remained significant in comparison with sham animals on days 1, 2, 5, 7 and 9 post-FCA ($P<0.05$, two-way ANOVA). However, thereafter the values returned towards basal levels and no further significant differences were observed ($P>0.05$, two-way ANOVA).

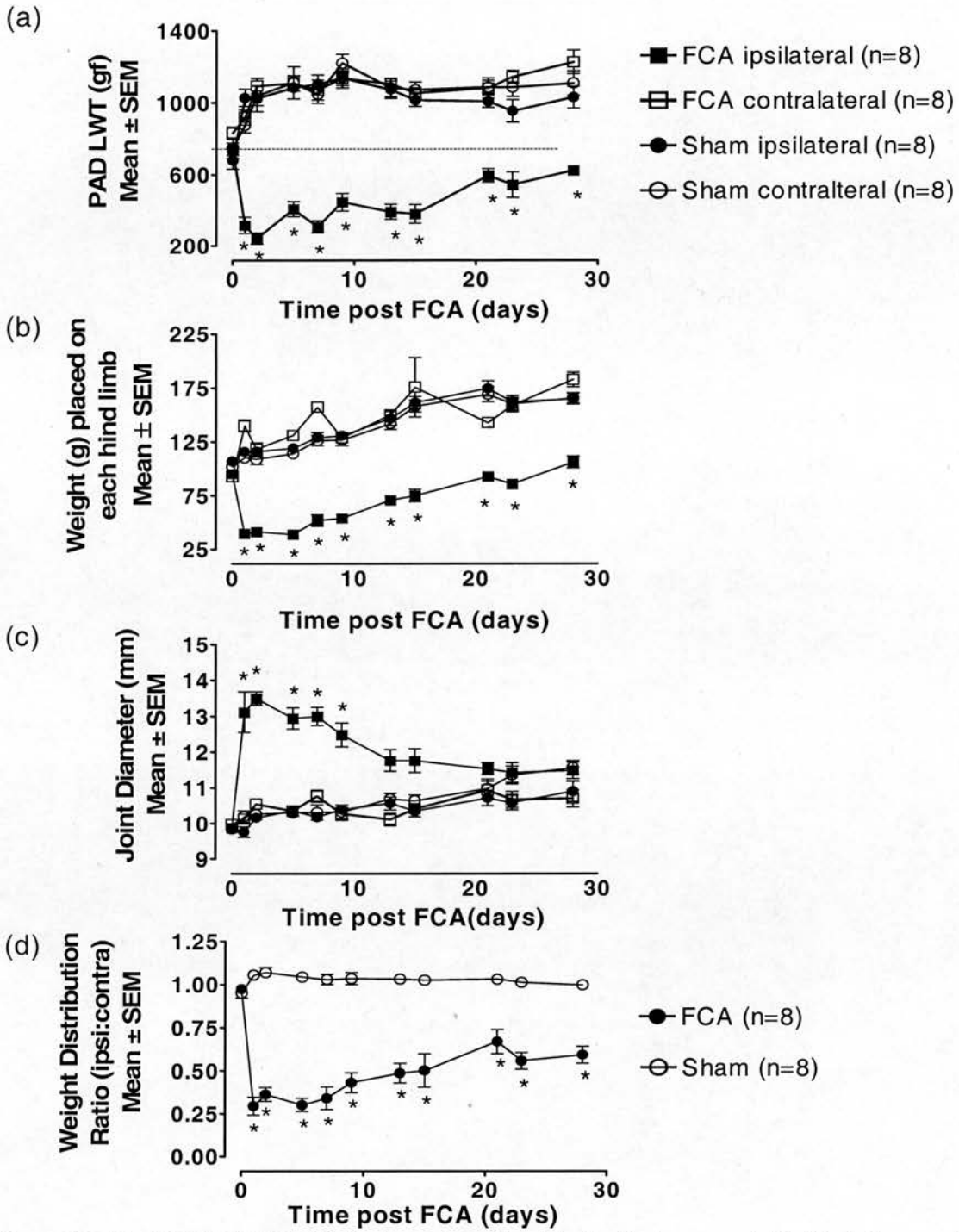


Figure 3.3. The (a) PAD LWTs, (b) absolute values of weight distribution on each hind limb (in grams), (c) knee joint diameters and (d) ratio of weight distribution between hind limbs of sham and FCA-injected (150 μg) rats over a 28 day pilot study. PAD detected hypersensitivity in FCA-injected animals, similar and comparable to that measured by the incapacitance tester. * represents statistical significance ($P<0.05$) comparing the groups at each time point using a two-way ANOVA and a Bonferroni post-hoc test.

3.3.2 PREDNISOLONE, MORPHINE AND CELECOXIB STUDY IN RATS

Pressure application device – PAD

There were no significant differences between the PAD ipsilateral LWTs of any groups prior to the induction of arthritis (n=56; $P>0.05$, Kruskal-Wallis). There were no differences between the average ipsilateral (1068 ± 23 gf) and contralateral (1037 ± 21 gf) LWTs of animals on day 0 (n=56; $P>0.05$, Wilcoxon; see Figure 3.4). Measurements were made on days 2, 4, 7 and 10 to determine that hypersensitivity was present prior to drug treatment. On each of these days the ipsilateral PAD LWTs of all FCA-injected animals (n=47; one rat was excluded from the study as it was found to have an existing limb injury that appeared to affect the behavioural measurements in this study) were significantly less than sham (n=8, $P< 0.05$, two-way ANOVA) and were not statistically different from values observed in the pilot study ($P> 0.05$, Mann Whitney).

On day 10 arthritic rats were randomly assigned into one of six treatment groups:

- celecoxib 15 mg kg^{-1} (n=8)
- morphine 3 mg kg^{-1} (n=8)
- prednisolone 1 mg kg^{-1} (n=7),
- prednisolone 3 mg kg^{-1} (n=8)
- prednisolone 10 mg kg^{-1} (n=8).

Drugs were administered daily between days 14 and 18, and behavioural assessments were made one hour after drug injections. AUC values were calculated for each individual animal to observe any drug induced reversal of hypersensitivity (analgesia) over the five day dosing period. Values were calculated using day 10 values as a baseline to observe changes from day 10 through until day 18. Results showed that PAD detected a significant analgesic effect, (*i.e.* an increase in LWT towards levels measured in sham animals) of morphine, celecoxib and

prednisolone (3 and 10 mg kg^{-1}; $P< 0.05$, Kruskal Wallis, compared with vehicle, see Figure 3.5 and 3.6). The dose of morphine used here produced no obvious motor effects at the time of behavioural testing which could interfere with the withdrawal reflexes.

Further PAD LWT measurements were taken on days 21, 24 and 28 after drug-treatment had ended. In all groups any analgesic effect of the compound previously administered was absent; there was no significant difference from the vehicle-treated group ($P>0.05$, Kruskal Wallis). No changes were seen in contralateral LWTs at any time during this study.

Incapacitance Tester – Weight Distribution

There were no significant differences between the weight distribution ratio of any animals prior to the induction of arthritis (n=56; $P>0.05$, Kruskal-Wallis; see Figure 3.4). The actual weights placed on each hind limb on this day were 64.0 ± 0.8 g and 63.0 ± 0.8 g on ipsilateral and contralateral limbs respectively. Measurements were made on days 2, 4, 7 and 10, to determine hypersensitivity was present prior to drug treatment regimes. On each of these days the weight distribution ratio of all FCA-injected animals (n=47) was significantly less than those of naive controls ($P< 0.05$, two-way ANOVA, n=8), and was not significantly different from those values in the pilot study ($P> 0.05$, Mann Whitney, see Figure 3.4).

On day 10, arthritic rats were randomly assigned into one of six treatment groups, as described above. Drugs were injected (s.c.) daily between days 14 and 18, and behavioural assessments were made one hour after drug injections. AUC values were calculated from daily measurements for the dosing period as described above and showed that the FCA-induced hypersensitivity was significantly attenuated by celecoxib (n=8), morphine (n=8) and prednisolone at 3 (n=8) and 10 mg kg^{-1} (n=8) ($P<0.05$, Kruskal Wallis, compared with vehicle), but not by 1 mg kg^{-1} (n=7) prednisolone ($P>0.05$, Kruskal Wallis; see Figure 3.5 and 3.6).

Further measurements were made on days 21, 24 and 28, after drug-treatment had ended. In all groups any analgesic effect of the compound previously administered was absent; there was no significant difference from the vehicle-treated group ($P>0.05$, Kruskal-Wallis).

Joint inflammation

There were no significant differences between the ipsilateral joint diameters of animals prior to the induction of arthritis (n=56; $P>0.05$, Kruskal-Wallis, see Figure 3.4). There were no differences between the average ipsilateral (8.81 ± 0.13 mm) and contralateral (8.97 ± 0.09 mm) knee joint measurements of animals on day 0 (n=56; $P>0.05$, Wilcoxon). Measurements were made on days 2, 4, 7 and 10 prior to start of the dosing period. On days 2, 4, and 7 the ipsilateral joint diameters of all FCA-injected animals (n=47) were significantly higher than those of sham (n=8, $P< 0.05$, two-way ANOVA: see Figure 3.4) and were not statistically different to values observed in the pilot study ($P< 0.05$, Mann Whitney).

Administration of morphine (3 mg kg^{-1}), celecoxib (15 mg kg^{-1}) and 3 or 10 mg kg^{-1} prednisolone had no effect on FCA-induced joint swelling. However, prednisolone at 1 mg kg^{-1} significantly reduced the average ipsilateral knee joint diameter ($P<0.05$, Kruskal-Wallis, compared with vehicle) during the dosing period (see Figure 3.5 and 3.6). No changes in contralateral joint diameters were observed in any group over this time period.

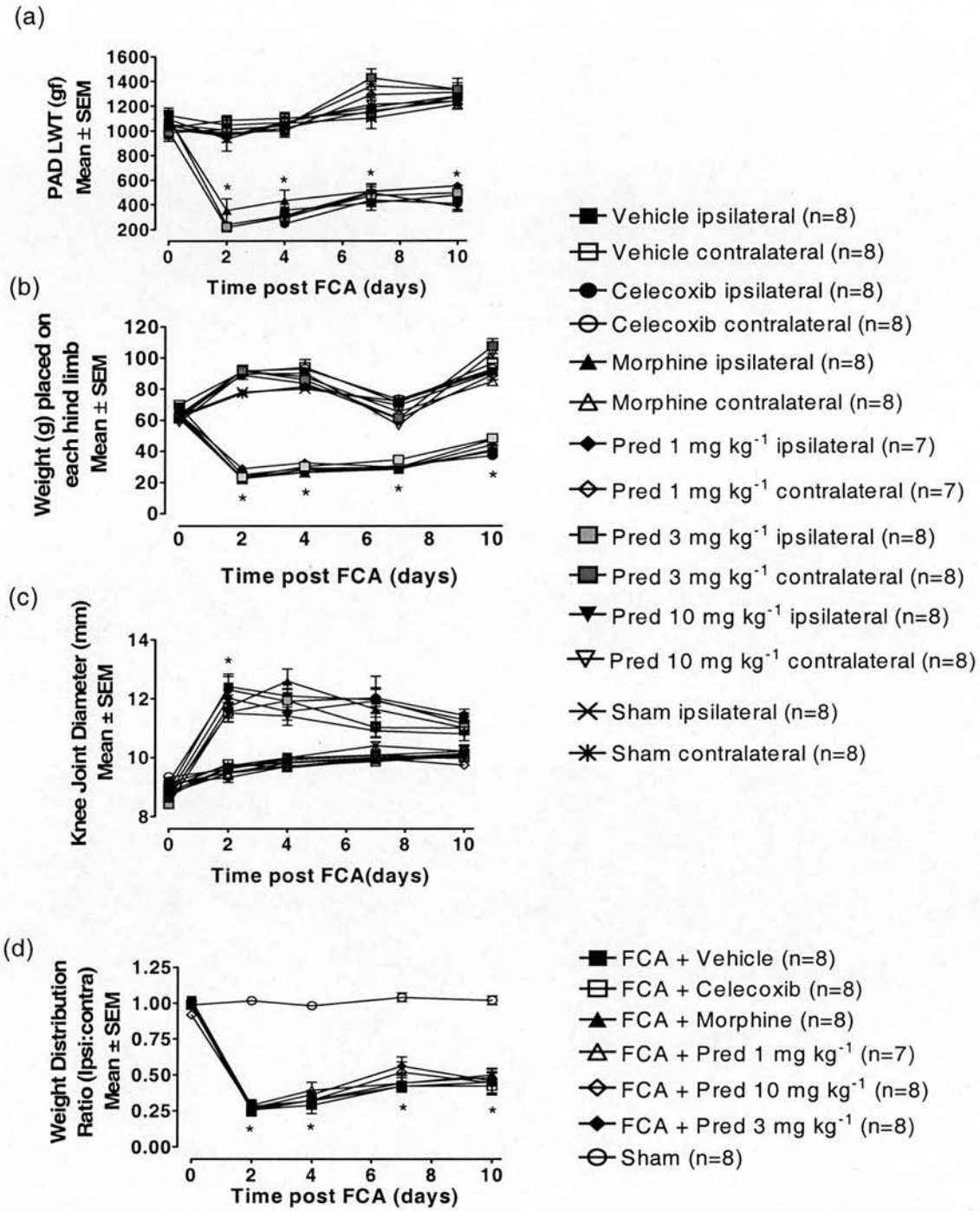


Figure 3.4 The (a) PAD LWTs, (b) absolute values in grams of weight placed on each hind limb, (c) knee joint diameters and (d) ratio of weight distribution between the hind limbs of sham and FCA-injected (150 μg) rats from day 0 up until and including day 10, prior to drug treatment. * represents statistical significance ($P<0.05$) comparing the groups at each time point using a two-way ANOVA and a Bonferroni post-hoc test.

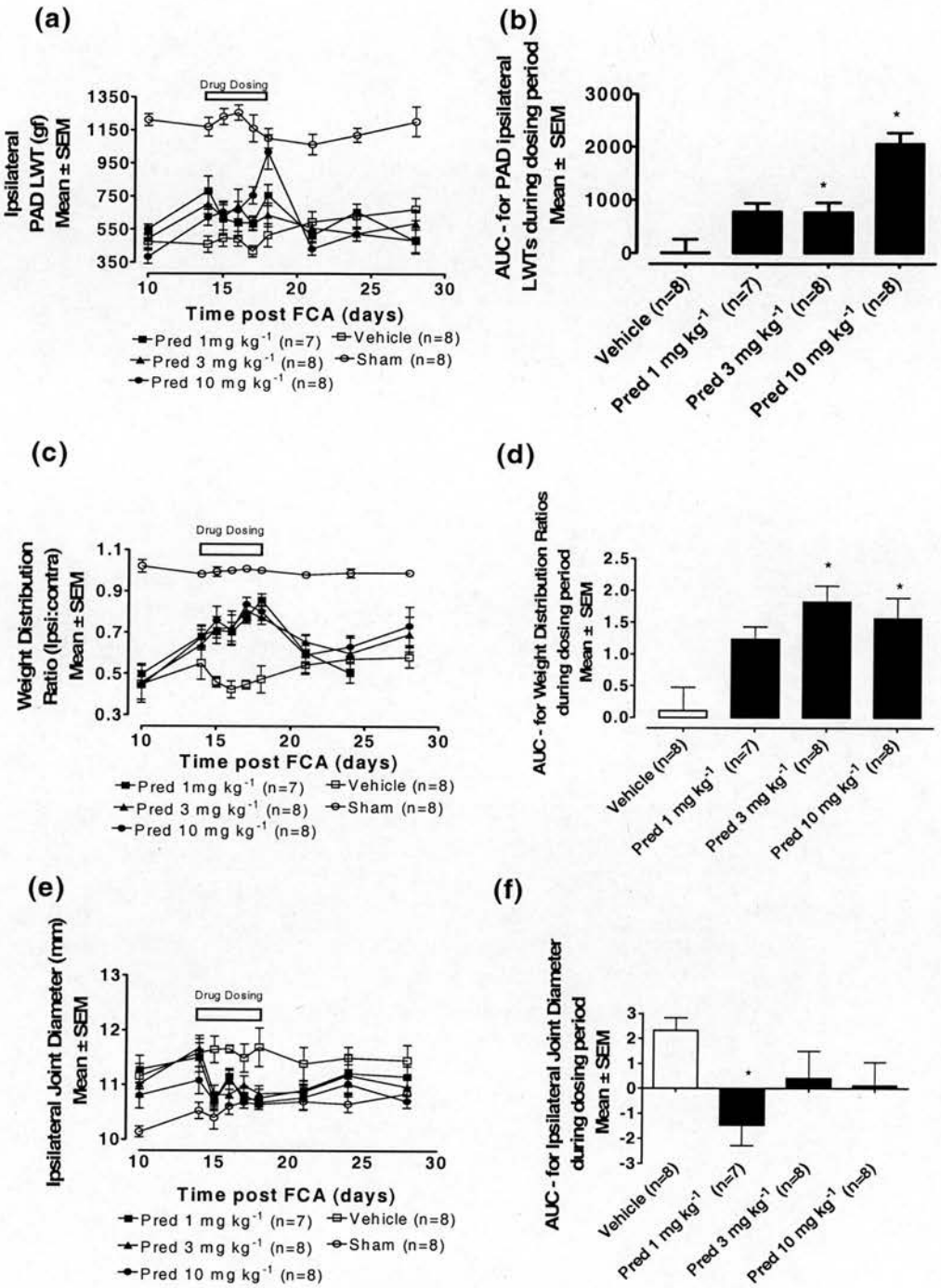


Figure 3.5 The (a) ipsilateral PAD LWTs, (c) weight distribution ratios and (e) ipsilateral knee joint diameters of prednisolone- and vehicle-treated arthritic and sham (control) rats and the corresponding AUC graphs during the dosing period (b, d and f respectively). The drug dosing period was between days 14 and 18 as indicated by the box on the graphs. Statistical analysis was carried out to compare the AUC values for the drug-treated group in comparison with vehicle; * represents statistical significance ($P<0.05$), determined by a Kruskal Wallis test.

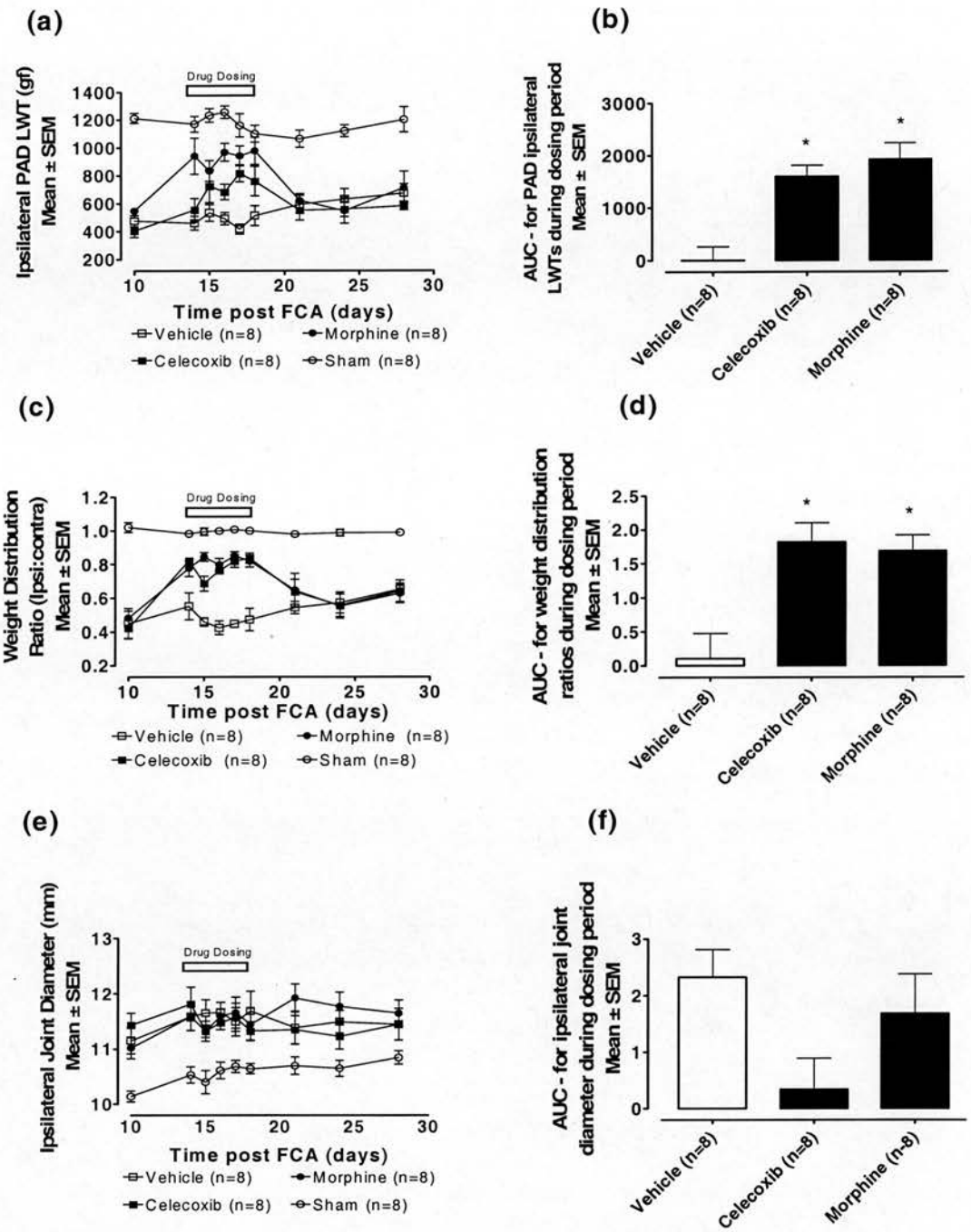


Figure 3.6 The (a) ipsilateral PAD LWTs, (c) weight distribution ratios and (e) ipsilateral knee joint diameters of morphine-, celecoxib- and vehicle-treated arthritic and sham (control) rats and the corresponding AUC graphs for the three readouts during the dosing period (b, d and f respectively). The drug dosing period was between days 14 and 18 as indicated by the box on the graphs. PAD was able to detect the analgesic effect in groups treated with both drugs compared with vehicle. Statistical analysis was carried out to compare the AUC values for the drug-treated group with that of vehicle-treated animals; * represents statistical significance ($P<0.05$) determined by a Kruskal Wallis test.

Correlation of PAD LWT with weight distribution readout

In order to determine the strength of the correlation between the results obtained from the two pain readouts, a Spearman's linear regression analysis was performed. The mean PAD ipsilateral LWT for each group was plotted against the mean ratio of weight distribution of the same group, with results from all experimental days included. A strong positive correlation between the results obtained from the two readouts was observed (see Figure 3.7; Spearman r =0.91; $P<0.0001$).

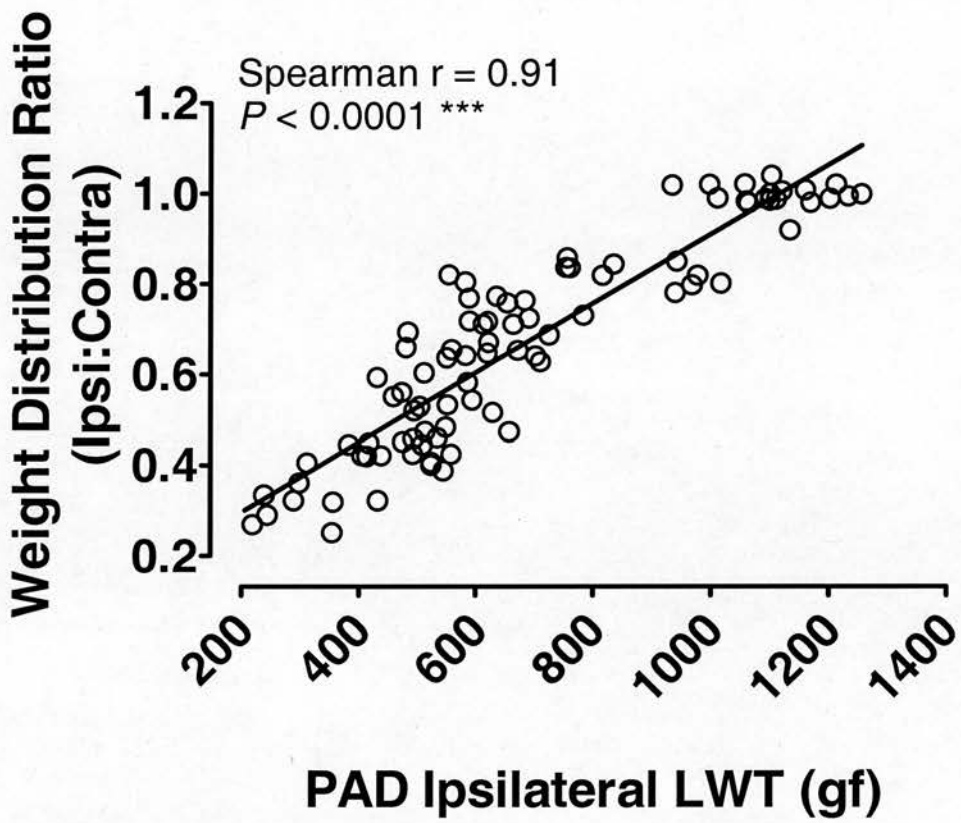


Figure 3.7. The correlation of the absolute ipsilateral LWT, as measured by PAD, and the ratio of the weight distribution between the hind limbs, measured by the incapacitance tester, in rats. There was a strong positive correlation between the two measures. The Spearman Linear Correlation factor was 0.91, which was highly significant ($P<0.0001$).

3.3.3 MURINE STUDY – FCA-INDUCED HYPERSENSITIVITY

Pressure application device - PAD

Prior to FCA-induced joint inflammation (on day 0) there were no significant differences between the groups' ipsilateral PAD LWT ($P>0.05$, Kruskal-Wallis); the values were 402 ± 16 gf (n=8), 400 ± 16 gf (n=8) and 437 ± 16 gf (n=8). There were no differences between the average ipsilateral (414 ± 10 gf) and contralateral (386 ± 14 gf) LWTs of animals, prior to arthritis induction (n=24; $P>0.05$, Wilcoxon).

The ipsilateral LWT of the FCA-injected groups decreased by 57% to 175 ± 6 gf (n=8) and 63% to 148 ± 16 gf (n=8) one day after injection. These LWTs were significantly lower than the sham group ($P<0.05$, Kruskal Wallis; see Figure 3.8) at this time point, indicating that PAD is able to detect hypersensitivity in mice following induction of joint inflammation. Following sham surgery, the average LWT of the control group dropped by 30% over the same 24 hour period to 307 ± 19 gf (n=8), a significant reduction from the day 0 value ($P<0.05$, Wilcoxon). However, this decrease was not sustained, and the value had increased towards basal values by day 3, and was no longer statistically different from baseline thresholds ($P>0.05$, Kruskal Wallis). Ipsilateral LWTs remained significantly lower in both FCA-injected groups in comparison with the sham animals on each day post-FCA ($P<0.05$, two-way ANOVA) up to and including day 10. Furthermore, ipsilateral PAD LWTs for FCA and vehicle-treated animals were significantly decreased compared with contralateral readings on each day post FCA.

Incapacitance Tester – Weight Distribution

The average baseline ratio of weight distribution between ipsilateral and contralateral limbs in mice was 1.0 ± 0.1 (n=24); this resulted from 9.9 ± 0.3 g and 9.8 ± 0.3 g on the ipsilateral and contralateral hind limbs respectively. There were no significant differences between the ratios of the three groups ($P<0.05$, Kruskal-Wallis).

There was a 36% reduction (to 0.65 ± 0.09; n=16) in FCA-injected animals' weight distribution ratio on day 1. This was significantly lower than that of sham animals ($P<0.05$, Mann Whitney, see Figure 3.8), which was 1.0 ± 0.03 (n=8), illustrating that the weight distribution readout is able to detect FCA-evoked hypersensitivity in mice. The ratio remained significantly reduced in FCA-injected animals compared with the sham group up until and including day 10 ($P<0.05$, two-way ANOVA), when drug treatment groups were assigned. There were no changes observed in the sham group at any time point in this behavioural readout ($P<0.05$, Kruskal-Wallis).

Joint Inflammation

Baseline knee joint diameters averaged 4.02 ± 0.02 mm (n=24) and 4.01 ± 0.02 (n=24) mm before FCA, these values were not significantly different ($P>0.05$, Wilcoxon). Intra-articular FCA evoked a significant swelling in all animals (n=16) compared with the sham group (n=8) on each day post FCA ($P<0.01$, two-way ANOVA; see Figure 3.8). No changes in knee joint diameter were seen in the sham group, or in the contralateral joints of FCA-treated animals at any time point ($P>0.05$, Kruskal-Wallis).

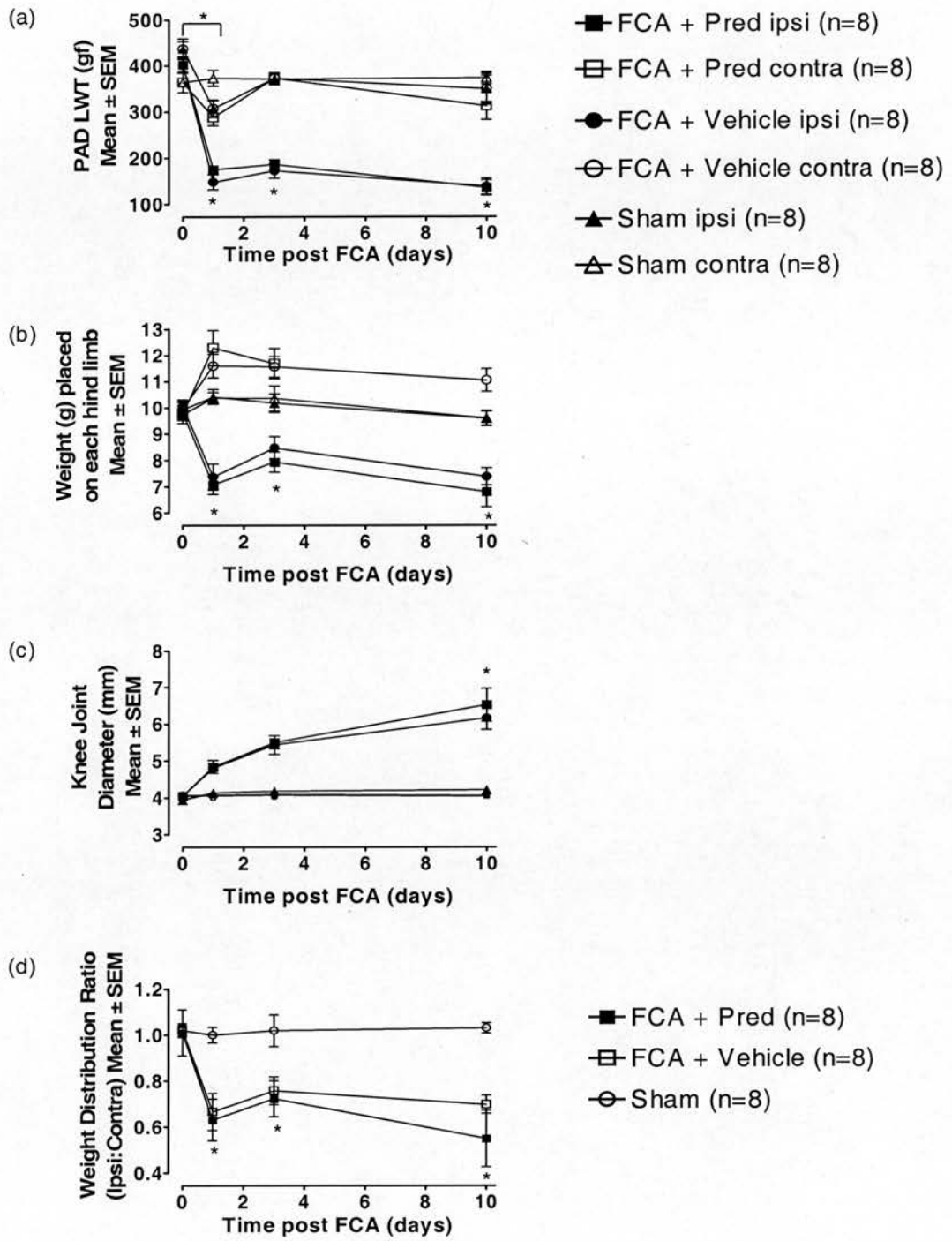


Figure 3.8 The (a) PAD LWTs, (b) absolute values in grams of weight placed on each hind limb, (c) knee joint diameters and (d) weight distribution ratios of sham and FCA-injected (200 μg) mice from day 0 up until and including day 10, prior to drug treatment. * represents statistical significance ($P<0.05$), determined by a Kruskal Wallis test.

3.3.4 MURINE STUDY – PREDNISOLONE INDUCED ANALGESIA

Pressure application device - PAD

FCA-injected mice had a significantly lower average PAD LWT on day 10 in comparison with the sham group, values were 137 ± 17 gf (n=16) and 349 ± 34 gf respectively (n=8; $P<0.01$, Mann Whitney). On day 10, FCA-injected mice were randomly assigned to one of two treatment groups:

- prednisolone (1 mg kg^{-1})
- vehicle.

Drugs were administered (s.c.) blind by Mr Iain Strickland, once a day between days 13 and 17 in a volume of 3 ml kg^{-1}; behavioural assessments were made one hour after drug administration. AUC values calculated for the prednisolone- and vehicle-treated groups between days 10 and 17 showed that prednisolone induced significant analgesia compared with the vehicle-treated group, as measured by PAD ($P<0.05$, Mann Whitney; see Figure 3.9). The largest individual reversal was observed on the final day of dosing, day 17, where the prednisolone-treated FCA group had an average PAD LWT of 310 ± 38 gf (n=8), which was 61% higher that LWTs in vehicle-treated mice (n=8). Following termination of drug-treatment, the ipsilateral LWT of prednisolone-treated animals returned to values near those of the vehicle-treated group. However, no significant difference between LWTs of FCA-injected and sham animals was detected ($P>0.05$, Kruskal Wallis). No changes were noted in contralateral LWTs in any group at any time during this study ($P>0.05$, Kruskal Wallis).

Incapacitance Tester – Weight Distribution

The average weight distribution ratio on day 10 for the FCA-injected mice was 0.64 ± 0.08 (n=16), which resulted from 7.9 ± 0.4 g on the ipsilateral joint and 11.2 ± 0.4 g on the contralateral side. The ratio was significantly lower in FCA-treated animals than the sham

group, in which the ratio of weight distribution between the two joints was 1.03 ± 0.02 ($P<0.05$, Mann Whitney; n=8). On day 10, FCA-injected mice were randomly assigned into one of two treatment groups; 1 mg kg^{-1} prednisolone or vehicle. Drugs were administered (s.c.) once a day between days 13 and 17; behavioural assessments were made one hour after injections. AUC values calculated for the prednisolone- and vehicle-treated groups between days 10 and 17 showed that there was significant analgesia as a result of prednisolone treatment, as determined by the incapacitance tester ($P<0.05$, Mann Whitney; see Figure 3.9). Following termination of drug-treatment, the weight distribution ratios of the drug-treated group returned to near those of the vehicle-treated animals, which was not significantly different than the values for sham animals.

Joint Inflammation

Prior to commencement of prednisolone treatment, average ipsilateral joint diameters in FCA treated animals was 6.53 ± 0.45 mm (n=16), in contrast values for control (sham) animals was 4.21 ± 0.11 mm (n=8). There were no significant differences between the groups ($P>0.05$, Mann Whitney). Prednisolone-treatment (1 mg kg^{-1}) caused a reduction in joint swelling; although the AUC values for this period were not significantly reduced compared with vehicle-treated mice, see Figure 3.9. Following termination of drug-treatment, the joint diameters of the drug-treated group remained lower than those of the vehicle-treated animals, however no statistical significance was evident ($P>0.05$, Mann Whitney). Furthermore, ipsilateral joint diameters in the FCA-injected animals were not significantly greater than sham values ($P>0.05$, Mann Whitney). No changes were observed in contralateral joint diameters at any time point during this study ($P>0.05$ Kruskal Wallis).

Correlation of the PAD LWT with the weight distribution readout

In order to determine the strength of the correlation between the results obtained from the two hypersensitivity readouts in mice, a Spearman's linear regression analysis was carried out. The PAD ipsilateral LWT for each group was plotted against the ratio of weight distribution of the same group, with results from all experimental days included. A strong positive correlation between the results obtained from the two different readouts was observed (see Figure 3.10; Spearman r =0.79; $P<0.0001$).

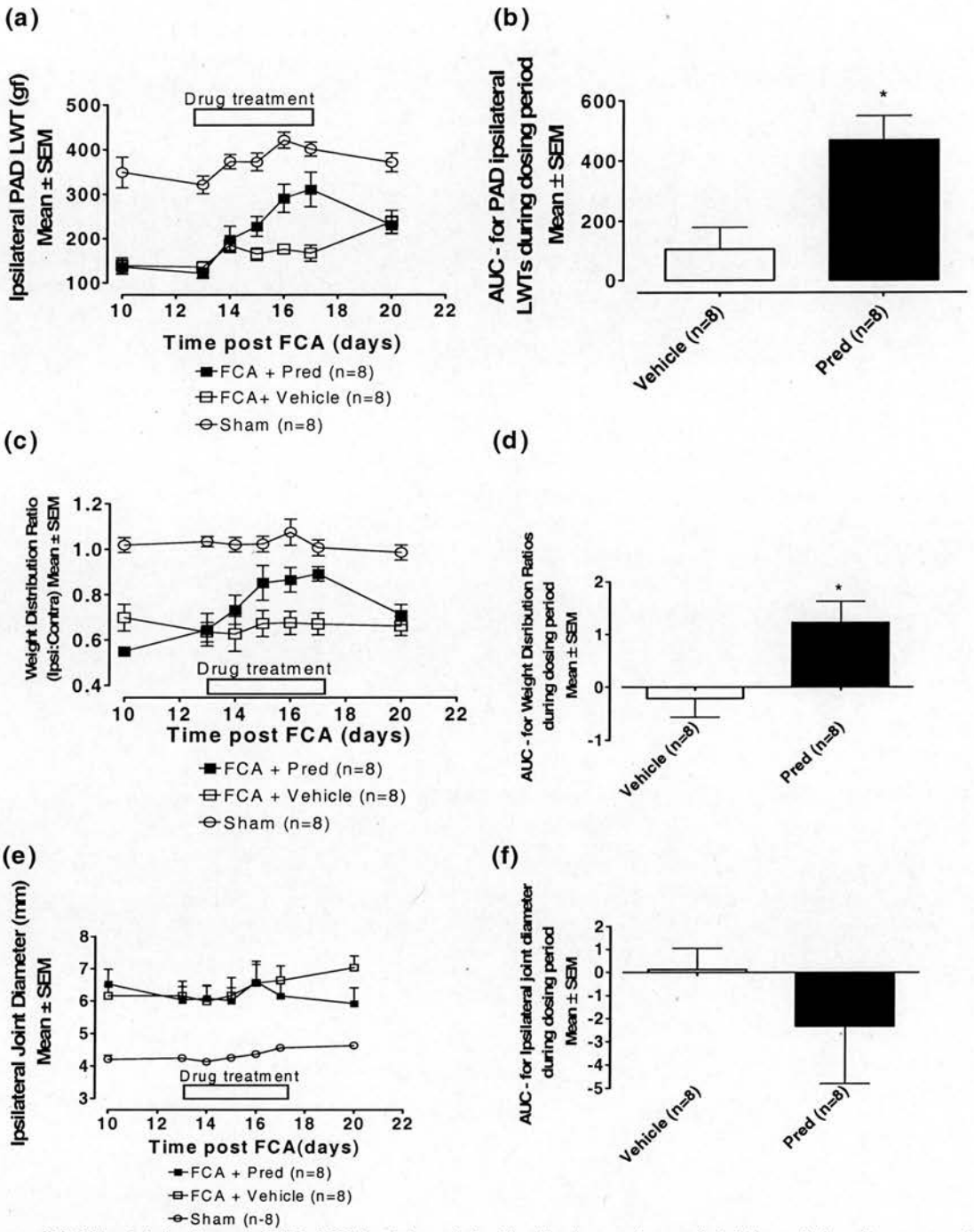


Figure 3.9 The (a) ipsilateral PAD LWTs, (c) weight distribution ratios and (e) knee joint diameters of FCA-injected (200 μg) or sham mice from day 10. Prednisolone (1 mg kg^{-1}) was administered (s.c.) on days 13 to 17 inclusive. AUC values for (b) PAD LWT, (d) weight distribution ratios and (f) joint diameters showed that prednisolone caused significant analgesia in both pain readouts. However, no significant difference in joint diameter was shown in this study as a result of prednisolone, at this dose. Statistical analysis was carried out on AUC data to compare the values for the FCA-injected groups to that of sham animals; * represents statistical significance (P<0.05), determined by Mann Whitney tests.

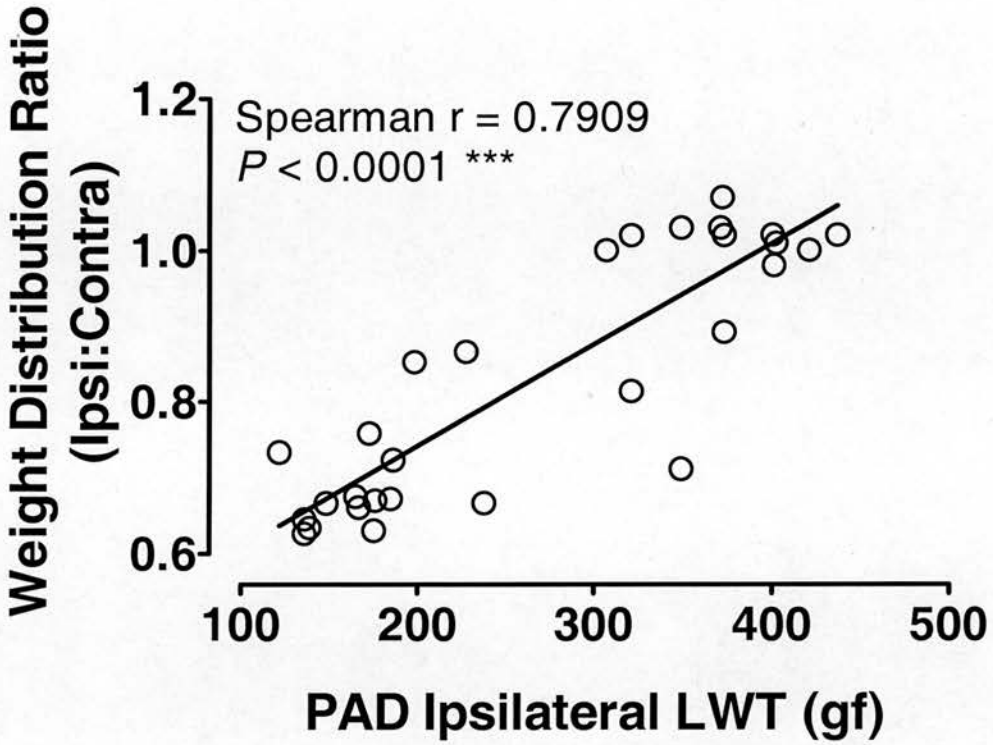


Figure 3.10 Correlation of the absolute ipsilateral LWT, as measured by PAD, and the ratio of the weight distribution between the hind limbs, measured by the incapacitance readout, in mice. There was a strong positive correlation between the two readouts; the Spearman Linear Correlation factor was 0.79, which was highly significant ($P<0.0001$).

3.4 DISCUSSION

A novel behavioural tool for assessing joint pain in two experimental rodent models of chronic inflammation has been developed and assessed. The results of these studies indicate that PAD provides a reliable, quantitative measurement of localised, FCA-induced mechanical hypersensitivity in the knee joint of rats and mice. PAD was also able to detect the analgesic action of prednisolone in mice and rats; and morphine- and celecoxib-induced analgesia in rats. Furthermore, a strong correlation between the weight distribution readout and the PAD measurements were made in both species, illustrating that PAD is a simple behavioural test that will be a valuable instrument for assessing hypersensitivity in the joint.

STIMULUS AREA AND NOCICEPTOR ACTIVATION

Pressure exerted onto the skin may activate nociceptive afferents in several tissues, depending on the surface area of the object used. Contact with a punctate object such as a needle may exclusively activate nerve endings in the skin, in particular C fibres. Because deformation of the skin can be achieved with very small forces (Garell *et al.*, 1996; Garnsworthy *et al.*, 1988; Khalsa *et al.*, 1997), these stimuli have little effect on afferents in deeper tissues. In contrast, a preferential activation of deep afferents is possible if pressure is exerted on a larger area of skin and the contact surface is rounded or padded (Treede *et al.*, 2002). According to experiments using topical local anaesthesia, the contribution of cutaneous afferents to pain evoked by blunt pressure is minimal (Kosek *et al.*, 1995). This evidence adds support to the use of PAD as a measure of pain in deeper tissues such as the joint itself, rather than overlying skin. This is in contrast to von Frey hairs, which as a punctate stimulus, activate skin afferents rather than those innervating the joint capsule and surrounding tissues.

Typical naive LWTs for rats and mice were approximately 1045 gf and 400 gf respectively. These values are considerably higher than those seen in the paw using calibrated forceps or the Randall and Selitto device (Baamonde *et al.*, 2004; Cook & Moore, 2006; Luis-Delgado *et al.*, 2006; Walker *et al.*, 2003), which is probably due to the difference in the size and gross anatomy of the paw and the knee joint, in addition to the surface area over which the pressure is applied using the different devices. Using the Randall and Selitto device paw withdrawal thresholds in naïve rats have ranged between 22 and 90 g, depending on sex, strain and size of the animals (Cicala *et al.*, 2000; Magari *et al.*, 2003). Others have modified the Randall and Selitto analgesymeter to measure pain thresholds of the ankle joint, and report much higher withdrawal thresholds, of around 150 g in naïve rats (Magari *et al.*, 2003). Studies using calibrated forceps to determine withdrawal thresholds of paws indicate much higher values, in the region of 500 g in naïve rats (Luis-Delgado *et al.*, 2006). Yu et al (2002) reported

vocalisation thresholds to compression force applied to the knee joint at approximately 1800 g in normal rats and falling to about 500 g following i.art FCA, using calibrated forceps, which again, is comparable to the values recorded in our study.

Neugebauer *et al.* (2002) report that pressure stimuli >1500 g 30 mm^{-2} applied to the knee joint with calibrated forceps was noxious, as it consistently evoked hind limb withdrawal reflexes in awake rats. When converted to gf mm^{-2}, our study which uses a circular disc with an area of approximately 50 mm^2, and showed LWTs in the region of 1000 gf in naïve rat joints, the values of pressure are 20 gf mm^{-2} or 600 gf 30 mm^{-2} for naïve rats. In mice the LWTs were also in the region of 20 gf mm^{-2} or 600 gf 30mm^{-2}, although the circular contact area was approximately 20mm^2. This indicates that the same pressure (20 gf mm^{-2}) is noxious in rats and mice when the joint is stimulated with PAD. However, the data from PAD is not routinely presented as a force per unit area (pressure), as it cannot be assumed that the entire surface of the disc is in contact with the joint, furthermore in inflamed joints the deformation of the joint onto the disc is different to that in naïve animals. PAD measurements are therefore more accurately expressed as a force, in gram force.

PAD *VS.* CLINICAL MEASURES OF JOINT PAIN

Clinically, joint pain is assessed either subjectively using the Ritchie Articular index or objectively using a dolorimeter (Langley *et al.*, 1983; Ritchie *et al.*, 1968). The Ritchie articular index allocates one of four grades of tenderness in patients' joints. Previous studies carried out in this laboratory used a similar subjective scale of joint hypersensitivity (Gauldie *et al.*, 2004). However, a quantitative measure which mimics the pressure dolorimeter would considerably improve assessments of experimental joint pain by providing an objective measure. The dolorimeter uses a gradually increasing force, applied in a perpendicular plane across the joint margin, to assess localised hypersensitivity of a human joint. The dolorimeter can be used on

different joint types and has been adopted in clinical studies assessing osteoarthritis of the knee (Ottillinger *et al.*, 2001) as well as the pain associated with fibromyalgia (Gracely *et al.*, 2003). Currently no objective measure of joint hypersensitivity is used to assess experimental arthritic joint pain in laboratory rodents. To that end, PAD was developed to align pre-clinical measures to those used clinically and help the translation of animal studies to human conditions.

PAD *VS.* ALTERNATIVE DEVICES TO MEASURE EXPERIMENTAL JOINT PAIN

Calibrated forceps have been used as a means of mechanical stimulation during electrophysiological recording (Li & Neugebauer, 2004; Neugebauer & Li, 2002), to measure knee joint or paw pain in experimental inflammation (Han *et al.*, 2005; Luis-Delgado *et al.*, 2006; Yu *et al.*, 2002) and to induce primary inflammatory hyperalgesia in deep tissues. These involved direct application of the forceps to muscle or to the knee joint of rats (Cui *et al.*, 1999; Skyba *et al.*, 2005). However, although the technique is reported to be an easy to use, reliable method of assessing nociceptive withdrawal thresholds, they also show a steady increase in paw withdrawal threshold over a 9 day period that was highly correlated to an increase in weight gain over this time (Luis-Delgado *et al.*, 2006). The authors suggest that the modification of pain sensitivity was due to increased size of the paw (Luis-Delgado *et al.*, 2006). This problem does not arise with PAD, although the LWT tended to increase over time as the animals gained weight, this wasn't significant, and no correlation to the body weight of the rats was observed. In addition, the weight distribution readout is also affected by the weight; although the ratio remains steady throughout the study, the actual weight placed on each hind limb increases as a result in the increase in the total weight of the animal over the time period of the study. For these reasons it is imperative to include a sham control group to compare all results to, to account for changes in PAD LWT and weight distribution data due to the gain in weight experienced by the animals over a chronic study such as those in this chapter.

An initial increase in LWT was noted in ipsilateral joints of naïve rats and contralateral joints of all rats, however, it was not significant and levels plateaued off after two days. In contrast, an initial decrease in LWT was observed in mice. This was seen in ipsilateral and contralateral joints in all three groups; however, it only proved to be a significant reduction from basal levels in ipsilateral knee joints, presumably as a result of the skin incision, which was necessary for intra-articular injection or sham surgery.

The authors in these studies reported several problems with the forcep style of device, including variation caused by the operator due to finger placement on the forceps, and inaccurate values of withdrawal thresholds, as the calibrated reading given is a function of the force applied by the experimenter and the resistance offered by the joint. PAD is calibrated directly across the force transducer and directly records the precise force placed across the knee joint, up until the point of withdrawal. As PAD uses a single force transducer worn on the experimenter's thumb, the amount of operator variation will only occur in the placement of the transducer on the knee joint of the animal being tested. However this is only a minor issue as the large surface area of the PAD thumb attachment removes potential inaccuracy in focusing on an exact point on the joint, which happens in the case of small forceps tips. A further advantage of PAD is the ability to change the size of the pressure application surface and the force range of the transducer, allowing the device to be used in several species as well as the possibility of assessing other joints and paws.

In this study, we chose to validate PAD by comparing the results to the current standard measure of hypersensitivity, the weight distribution readout. However, PAD is designed to measure evoked mechanical hypersensitivity of the joint, whereas the incapacitance tester is typically considered a measure of spontaneous pain. However, due to the fact that the animals are required to stand in the Perspex box and are moved from their home-cage, the incapacitance tester cannot truly be described as a measure of spontaneous pain, and most likely incorporates

aspects of evoked and ongoing, spontaneous pain, as evidenced by the strong degree of correlation between the two measures in this thesis. Therefore, it is possible to confidently validate a new device for measuring evoked mechanical hypersensitivity, in this case PAD, against the weight distribution readout, given that there is no other current standard measure for this parameter. PAD is thus an easy to use device allowing us to perform rapid, reproducible measurements. The absence of a significant difference between the ipsilateral and contralateral LWT in naïve animals, together with the low variability, indicates that PAD could use the contralateral joint as an internal control, an idea originally proposed by Randall and Selitto (1957).

DETECTION OF HYPERSENSIVITY AND SUBSEQUENT ANALGESIA

PAD was able to detect FCA-induced hypersensitivity in both mice and rats, observed as a decrease in LWTs of around 60% and 40% respectively, compared with the basal levels in normal joints. This hypersensitivity in the acute stage of the arthritis was mimicked by the significant inflammation observed following FCA injection. The ability of PAD to detect FCA-evoked hypersensitivity provides an excellent experimental model in which to test the analgesic properties of novel compounds over a period of stable inflammation between days 13 and 18 post-FCA.

Prednisolone, a standard positive control in drug screening (Pyne *et al.*, 2004), was studied over a five day period to check whether the drug would lead to a resolution of FCA-induced hypersensitivity as a correlate to the analgesia we would expect to see in man (Pyne *et al.*, 2004). PAD detected prednisolone analgesia, in both species over the five days of dosing, which was abolished after dosing ceased. PAD also showed significant reversal of evoked mechanical hypersensitivity in arthritic animals treated with the opioid morphine and the

cyclooxygenase-2 inhibitor, celecoxib, which was comparable to that seen in the weight distribution readout.

Results in mice and rats show strong correlations between the two behavioural assessments. These results suggest that both techniques have the ability to detect a joint hypersensitivity, which can be attenuated using gold-standard analgesics. PAD has the added advantage of assessing the hypersensitivity of the joint at the site of the inflammation, in a similar fashion to the clinical dolorimeter (Langley *et al.*, 1983)

CONCLUSIONS

In conclusion, the present study shows that PAD provides a novel, accurate behavioural tool for detecting a localised primary mechanical hypersensitivity in two animal models of chronic inflammatory joint pain. PAD is the first tool designed to measure both primary and secondary hypersensitivity objectively. It can be used in various experimental pain models and could be extended for use in other species. Results from these pilot studies suggest PAD will aid the screening of novel analgesics designed to improve chronic inflammatory pain.

Chapter 4 – Development and validation of a joint perfusion system to enable assessment of inflammatory mediator and cell content of rat knee joints

4.1 INTRODUCTION

Inflammatory joint diseases such as RA are regulated by complex interactions involving many mediators, including prostanoids and cytokines (see Chapter 1). The infiltration of cells into the synovial tissue and joint space is another key characteristic of synovitis, which combined with the release of inflammatory mediators and degradative enzymes, eventually leads to cartilage and bone destruction (for reviews see Sweeney & Firestein, 2004).

Measuring the levels of these mediators in the synovial fluid from patients can provide information about the underlying pathophysiology of joint disease (Kubota *et al.*, 1998), for example the severity and current activity (Alstergren *et al.*, 1998; Chang & Israel, 2005; Rooney *et al.*, 1990) as well as inter-individual variations in disease (Ulfgren *et al.*, 2000) and effectiveness of drug-treatments (for review see Barrera *et al.*, 1996). Furthermore changes occurring in the synovial fluid can be used as biomarkers of disease, as has already been demonstrated in RA for plasma levels of inflammatory proteins (Eastgate *et al.*, 1988; Houssiau *et al.*, 1988).

Fluid samples from human joints have been analysed for inflammatory mediator content from both healthy volunteers and patients with joint diseases. These studies revealed the importance of particular cytokines, including TNFα, IL1β, and IL6, which are now targets for DMARDs (for review see Christodoulou & Choy, 2006; Zwerina *et al.*, 2005). Furthermore, increases in virtually all the prostanoids were detected in synovial fluid from human joints (Egg, 1984; Trang *et al.*, 1977), but notably PGE_2, which has been associated with erosion of bone and cartilage in RA (Dayer *et al.*, 1976; Fulkerson & Damiano, 1983; Robinson *et al.*, 1975a; Robinson *et al.*, 1975b).

Although some studies have investigated the fluid from joints, most research has been focused on the inflammatory mediators within the synovial membrane. One reason for this is the technical difficulty of trying to measure cytokine levels in such a viscous material as synovial fluid. Several studies have assessed cytokine gene expression levels in the

synovial membrane, rather than the actual protein content, both in human clinical samples (Firestein *et al.*, 1990; Wagner *et al.*, 1997) and in animal models of arthritis (Patten *et al.*, 2004; Rioja *et al.*, 2004; Thornton *et al.*, 1999). In addition, PGE synthase, the enzyme responsible for the conversion of COX-derived PGH_2 to PGE_2 has been detected in synovial tissues of patients with RA (Westman *et al.*, 2004).

The early time course for the release of key mediators cannot be determined using human synovial fluid samples, as patients rarely report to the clinic until the disease has progressed and is causing chronic pain and swelling (Cohen *et al.*, 1995). Even then, repeated sampling from individuals is difficult and most patients are prescribed drugs, to improve their symptoms and quality of life, which interfere with inflammatory regulatory processes and cytokine expression. Therefore animal models of joint disease can be used to study the early events in the inflammatory process and the effects of drugs on inflammatory markers can be measured under controlled conditions.

Rat adjuvant-induced unilateral arthritis is a well established RA disease model (Bileviciute *et al.*, 1993; Billingham, 1987; Mapp *et al.*, 1993) and use of this model has facilitated the understanding of the time-course of the pathology in clinical RA (see Section 1.5.1). The model closely mimics the pathology of human RA, including histopathological changes, cell infiltration, hypersensitivity and swelling of the affected joint (Donaldson *et al.*, 1993; Pelegri *et al.*, 1995; Wilson *et al.*, 2006). Previous studies in animal models of joint inflammation have investigated the time course of cytokine protein or gene expression using homogenates of entire joints or paws from rats *post mortem* (Magari *et al.*, 2003; Marinova-Mutafchieva *et al.*, 1997; Patten *et al.*, 2004; Rioja *et al.*, 2004; Smith-Oliver *et al.*, 1993; Thornton *et al.*, 1999). A major limitation of these studies is that such sampling always includes bone, synovial tissue, synovial fluid and surrounding muscles and connective tissue, which will not allow the origin of any analytes to be determined. Others have surgically dissected and lavaged knee joints in order to collect the synovial fluid from dead animals (Keeble *et al.*, 2005; Singh *et al.*, 1997; Vale *et al.*, 2004). As yet, no studies

appear to have been carried out by perfusing saline through the intact joint space and collecting samples from intact anaesthetised animals.

The initial aim of this study was to develop a perfusion method to sample the synovial fluid and to use it to study the effects of a joint insult on the temporal release pattern for PGE_2, IL1β and TNFα concentrations and inflammatory cell infiltrate levels in the joint. During this study joint perfusates were assayed for particular cytokines and inflammatory cells from normal and arthritic rats.

4.2 METHODS

4.2.1 STUDY DESIGN

Anaesthetic effects

In order to determine the effect of anaesthetic agents on inflammatory mediators in joints, control experiments were performed. Firstly, five naive rats were anaesthetised with urethane (ethyl carbamate; 0.6 ml 100 g^{-1} body weight; 25% w v^{-1} solution; single i.p. injection) and five with sodium pentobarbital (1 ml kg^{-1} body weight; 60 mg ml^{-1} solution; single i.p. injection maintained with i.v. 375 μl hr^{-1} 20 mg ml^{-1} solution). Animals were placed on automated heating blankets connected to a thermistor probe inserted in the rectum to maintain their body temperature at 37°C. No other procedures were carried out for seven hours, at which point perfusion needles were inserted into left and right knee joints and a 250 μl sample collected. The samples were frozen immediately at -20°C, stored in a freezer and later assayed using the Luminex assay.

Effect of the perfusion needles in the joint space

To investigate whether the perfusion needles had any effects on synovial cytokine content, six animals were anaesthetised with urethane (as described above) and the perfusion needles inserted into both knee joints once the animals were fully anaesthetised. The needles

were held in position for seven hours, and then a 250 μl sample was collected. The sample was frozen and stored at -20°C and later assayed using ELISAs.

Effect of joint perfusion on the concentration of analyte

Two naïve rats were anaesthetised with urethane (as described above) and a basal joint perfusate sample taken immediately. Recombinant rat IL1β (100 μl of 10 ng ml^{-1}) was infused over one minute. One hour later a second sample was taken; this was repeated hourly until six or seven hour's post-IL1β infusion. The samples were frozen and stored at -20°C and later assayed for IL1β content using an ELISA to determine whether the sample contained the same amount of IL1β that was initially infused.

IL1β and TNFα in normal and FCA-injected joints

Basal samples from normal animals (n=16) were compared with basal samples from rats treated with FCA (i.art) 14 days earlier (n=14). All animals were anaesthetised using urethane, as described above. Samples were collected and then frozen and stored at -20°C for later analysis with ELISAs for rat TNFα and IL1β.

PGE_2 expression over a 21-day FCA time course

Basal samples were taken from rats 1, 3, 5, 7, 10, 14, 16, 18 and 21 days post-FCA (n=4 per day, n=36 in total) and compared with PGE_2 baseline values from naïve rats (n=4). Samples were frozen at -20° C and later assayed using an ELISA for PGE_2.

Total inflammatory cell counts

Joints were perfused with 3.15% sodium citrate in sterile saline and the perfusate collected from five naïve rat knee joints, five FCA-injected (150 μg; 14-day post FCA) ipsilateral and contralateral joints and three FCA-injected (500 μg; 14-day post-FCA)

ipsilateral and contralateral joints. Undiluted samples were viewed by light microscopy in a haemocytometer. If red blood cells or a high number of inflammatory cells were present, samples were diluted in saline, with added Zappoglobin, as per the manufacturer's instructions (1 drop per 20 ml).

4.3. RESULTS

4.3.1 ANAESTHETIC EFFECTS

Samples from naïve animals (n=5) which received no treatment during seven hours of urethane anaesthesia, showed a slight trend towards increased levels of inflammatory mediators. However, the increases were not statistically significant for IL1α, IL1β, IL2, IL4, IL6, IL10, GM-CSF, IFNγ, or TNFα compared with samples obtained immediately after induction of anaesthetic ($P>0.05$, Kruskal Wallis; see Table 4.1). In contrast, those animals anaesthetised with pentobarbital (n=5), had significantly higher levels of GM-CSF and TNFα ($P<0.05$, Kruskal Wallis) after seven hours, in comparison with naïve joints, see Table 4.1.

	IL1α	IL1β	IL2	IL4	IL6	IL10	GM-CSF	IFNγ	TNFα
Basal Mean ± SEM (pg ml^{-1}; n=10)	0.9 ± 0.6	1.2 ± 1.2	0.2 ± 0.2	0.3 ± 0.3	3.2 ± 3.2	1.9 ± 1.3	0.2 ± 0.2	0.2 ± 0.2	0.3 ± 0.3
Urethane Mean ± SEM (pg ml^{-1}; n=5)	2.6 ± 1.1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.0 ± 0.6	0.3 ± 0.3	36.6 ± 20.9
Pentobarbital Mean ± SEM (pg ml^{-1}; n=8)	1.4 ± 0.7	0.2 ± 0.2	0.1 ± 0.1	0 ± 0	0 ± 0	6.2 ± 6.2	1.7 * ± 0.4	0.1 ± 0.1	44.2 * ± 21.6

Table 4.1. The concentrations (pg ml^{-1}) of nine cytokines in normal (untreated) rat knee joint perfusate samples from animals anaesthetised for seven hours with either urethane (n=5) or pentobarbital (n=8), or samples taken immediately after urethane anaesthesia (basal; n=10). Kruskal Wallis tests were performed to determine the differences between the cytokine concentrations in joints of rats anaesthetised with each anaesthetic and basal levels. Pentobarbital anaesthesia caused a significant elevation of GM-CSF and TNFα; statistical significance ($P<0.05$ compared with basal levels) indicated by *.

4.3.2 EFFECT OF THE PERFUSION NEEDLES IN THE JOINT SPACE

Joint perfusion samples from knee joints in which the perfusion needles had been in place for seven hours in anaesthetised rats (n=6) showed increased levels of TNFα and IL1β, as measured by ELISA (see Figure 4.1). However, the levels were not significantly different from basal samples taken from the same rats immediately after needle insertion ($P>0.05$, Mann Whitney). Furthermore, it was different rats that had increased levels of the two proteins.

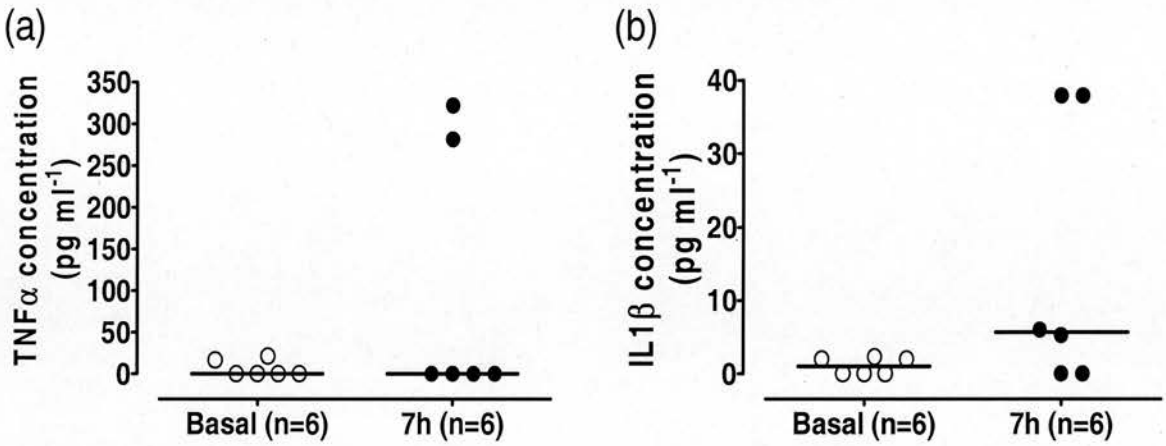


Figure 4.1 The concentrations of (a) TNFα and (b) IL1β measured in perfusates from joints immediately after needle insertion (basal) and seven hours (7h) later. Cytokines were assayed using an ELISA, and although there was an apparent increase in both proteins in two samples at the 7h time point; the median value (n=6) was not significantly different to basal samples ($P>0.05$, Mann Whitney). The horizontal lines on the graphs represent the median values in each group.

4.3.3 EFFECTS OF PERFUSION ON THE CONCENTRATION OF ANALYTE

A study was performed to determine if any of the infused solution leaked from the joint space prior to withdrawal of samples. Recombinant rat IL1β (1000 pg in 100 μl) was infused into the joint and samples were collected hourly. In both cases the full amount (1000 pg) administered was recovered in the first two samples. In fact, more than the administered amount of IL1β was recovered (see Table 4.2).

	Animal 1		Animal 2	
	IL1β concentration (pg ml^{-1}; 250 µl)	Amount of IL1β (pg in 250 µl)	IL1β concentration (pg ml^{-1}; 250 µl)	Amount of IL1β (pg in 250 µl)
1 hr	2000	500	2000	500
2 hr	2000	500	2000	500
3 hr	200	50	356	89
4 hr	544	136	216	54
5 hr	350	87.5	210	52.5
6 hr	458	114.5	200	50
7 hr	318	79.5	-	-
Total (pg) % of inj dose	1467.5 (147% of adminstered dose)		1245.5 (125% of administered dose)	

Table 4.2 The IL1β concentrations in each 250 µl sample collected, up to six or seven hours post-infusion of IL1β (1000 pg) . The amount of IL1β protein in each sample was calculated and summed, to show that little or no leakage from the joint space occurred; in fact, more IL1β was present in the perfusate in comparison with the amount initially injected.

4.3.4 TNFα AND IL1β IN NORMAL AND FCA-INJECTED JOINTS

Fourteen days after rats received FCA (150 µg; n=14), the ipsilateral (injected) joint showed significantly higher levels of both TNFα ($P<0.05$, Mann Whitney) and IL1β ($P<0.05$, Mann Whitney) compared with samples from naïve joints (n=16), as measured by ELISA, see Figure 4.2.

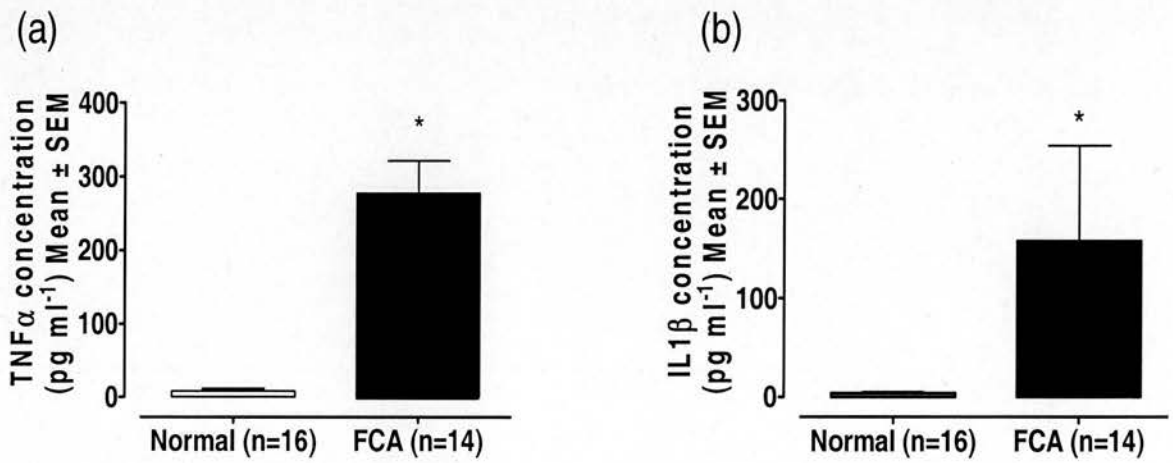


Figure 4.2 The concentrations of (a) TNFα and (b) IL1β in normal (untreated; open bar) and FCA-injected (closed bar) joints 14 days after arthritis induction. There were negligible levels of either protein in naïve joints, but a significant increase in their expression was seen in inflamed joints ($P<0.05$, Mann Whitney); statistical significance ($P<0.05$) represented by *.

4.3.5 PGE_2 EXPRESSION OVER A 21-DAY FCA TIME COURSE

FCA (150 μg) caused a biphasic expression of PGE_2 within the ipsilateral joint cavity, see Figure 4.3. Basal levels, measured in naïve joints, were 1865.2 ± 214.0 pg ml^{-1} (n=4). The initial peak of expression occurs at day 3 and the second peak at day 10. However, none of the PGE_2 levels at any time point proved to be significantly elevated in comparison with basal level (P>0.05, Kruskal-Wallis; n=4 on each day).

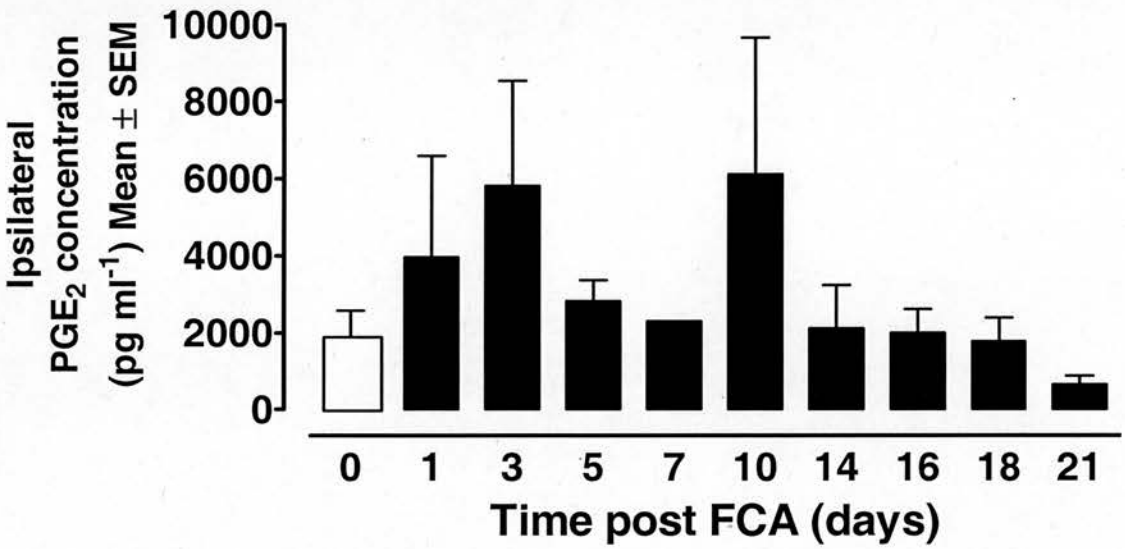


Figure 4.3 The concentration of PGE_2 in FCA-treated (150 µg; closed bar) and normal (untreated; open bars) joints (n=4 on each day). There was a biphasic expression pattern, peaking on days 3 and 10, although the increase was not statistically significant overall (P>0.05, Kruskal-Wallis).

Contralateral joint levels of PGE_2 also increased following i.art FCA, although at each time point they remained lower than ipsilateral joint levels. Prior to induction of joint inflammation, the levels of PGE_2 in contralateral joints was 1486 ± 687.1 pg ml^{-1}; this increased to a peak of 3853.0 ± 1310.4 pg ml^{-1} one day after FCA. Further measurements were less than basal measurements, and none of the contralateral PGE_2 levels were significantly different from day 0 readings (P>0.05, Kruskal Wallis).

4.3.6 TOTAL INFLAMMATORY CELL COUNTS

Total inflammatory cell counts from normal animals and those injected with FCA (n=7) 21 days prior to sampling are shown in Figure 4.4. Normal joints (n=5) had no inflammatory cells detectable, whereas all other samples had measurable levels. However, only the high dose FCA ipsilateral (500 μg; n=3) joints proved to have a significantly greater number of inflammatory cells ($4.8 \pm 0.06 \times 10^6$ cells ml^{-1}) than normal joints ($P<0.05$, Mann Whitney). The ipsilateral joint contained a higher number of inflammatory cells than the contralateral joint in rats injected with both doses of FCA. However, ipsilateral and contralateral joints' inflammatory cell counts in rats injected with the higher dose of FCA had increased levels in comparison with those injected with the low dose FCA.

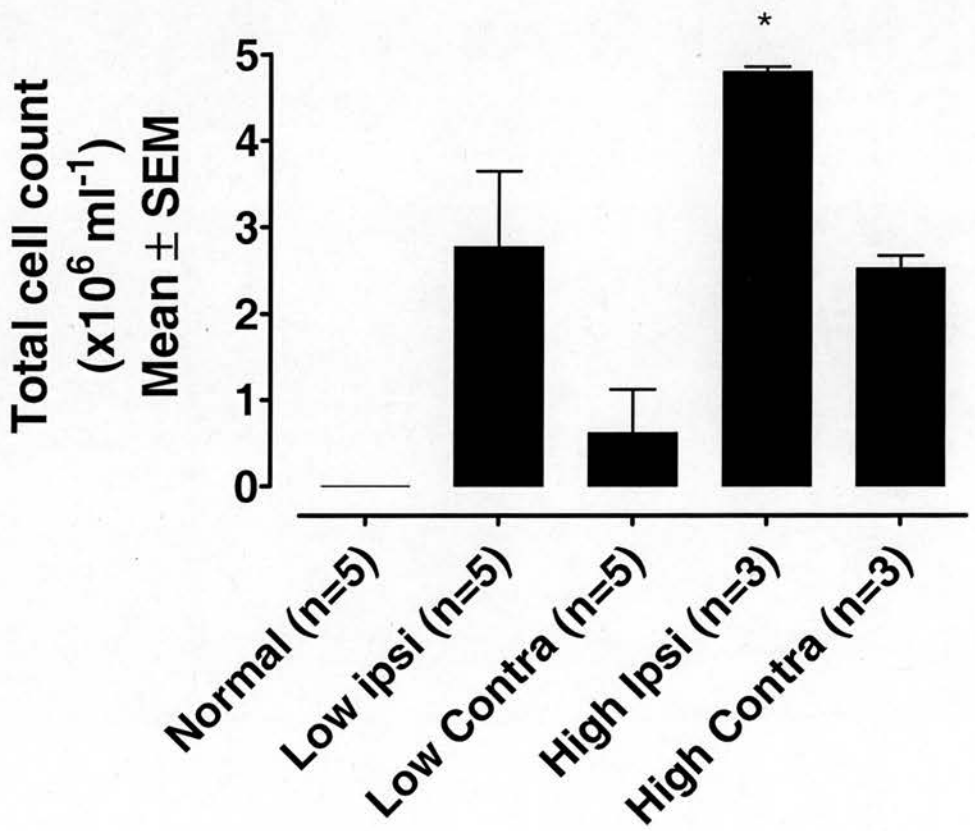


Figure 4.4. The effects of low (150 µg) and high (500 µg) dose FCA on total inflammatory cell counts from joint perfusates. Naïve joints contained no detectable inflammatory cells (0), whereas all other joints contained increased levels, although only high dose ipsilateral joints proved to have a significantly greater number of inflammatory cells in comparison with normal joints ($P<0.05$, Mann Whitney); statistical significance represented by *.

4.4 DISCUSSION

The aim of this study was to develop a method for sampling synovial fluid from the knee joints of anaesthetized rats. The technique was validated by determining whether an inflammatory response was evoked by the experimental protocol, including the anaesthetic and the perfusion needles themselves. The efficiency of the system was also investigated, *i.e.* whether any of the infused solution leaked from the joint space. Finally, the novel perfusion technique was used to quantify inflammatory cell and mediator content within the rat synovial cavity.

This technique proved to be reliable and consistent when perfusing the joint cavity and regular volumes of sample were easily collected. There were no problems with measuring inflammatory protein content due to high sample viscosity, a problem previously reported with the analysis of synovial fluid. This technique is therefore a valuable addition to protocols which use homogenates of entire joints to assess inflammatory mediator content.

It was established that the choice of anaesthetic may play a role in initiating an inflammatory response within the knee joint. Urethane, a hypnotic anaesthetic agent commonly used for laboratory animals did not affect levels of any of the mediators measured over a seven hour period. In contrast, pentobarbital (pentobarbitone), a short-acting anaesthetic which must be maintained by i.v infusion, therefore requiring further surgical preparation of the animal, was associated with increases in GM-CSF and TNFα after continuous administration during the day. I suspect that there was neither a pro-inflammatory effect of pentobarbital nor an anti-inflammatory effect of urethane, merely that the extra surgical preparation required to cannulate a vein for continuous administration of pentobarbital to maintain the level of anaesthetic required for the duration of the perfusion lead to a systemic inflammatory response, which was subsequently detected in the joint perfusate samples from rats anaesthetised with pentobarbital. In order to determine if this was the cause of the increased levels of TMFα and GM-CSF in basal samples from pentobarbital-anaesthetised rats, a further study could be conducted in which the

pentobarbital anaesthetic was administered regularly via i.p. injections rather than intravenous delivery. It was therefore decided to use urethane for joint perfusion experiments, given that it provides an extended period of anaesthesia with minimal physiological changes (Sapru & Krieger, 1979), without the need for invasive surgical preparation. Furthermore, pentobarbital can cause respiratory depression in rats, whereas urethane causes minimal cardiopulmonary disturbances (Field *et al.*, 1993; Wixson *et al.*, 1987). Hypoxia, under pentobarbital anaesthesia, may cause release of inflammatory mediators, as seen in this study.

Once it had been established that urethane had no adverse effects on the system, it was necessary to evaluate any inflammatory component resulting from insertion of the needles and the presence of the needles in the joint over seven hours. It was observed that a few rats showed increased TNFα or IL1β levels, but the changes occurred in only 20% of animals and were not statistically significant.

This study has demonstrated that very little, if any, solution perfused into the joint is lost into the surrounding tissue, and that the perfusate can all be recovered through the effusion tubes. This was confirmed by injection of Evans blue dye into the joint cavity and later dissection of the tissue. Although this study was not designed to show the effects of the exogenous inflammatory protein on the joint, IL1β caused *de novo* release of natural IL1β, as shown by the fact that levels of IL1β in the perfusate were 20 - 40% greater than the initial dose administered.

Adjuvant-induced arthritis is a widely used model of inflammatory joint disease, and it was therefore important that samples collected via perfusion of the joint contained detectable cytokine levels in comparison with naïve joints and FCA-injected inflamed joints. Levels of both the cytokines measured in this study, IL1β and TNFα, increased substantially 14 days after the initial insult to the joint. A 34-fold elevation of TNFα levels, and a 42-fold increase for IL1β levels occurred. This is in agreement with previous studies using homogenised rat joints (Smith-Oliver *et al.*, 1993; Szekanecz *et al.*, 2000).

Other key mediators which play vital roles in the pathophysiology of joint inflammation, such as PGE_2 showed a trend toward increased expression levels. . Previously PGE_2 had been identified in other inflammatory models, such as whole homogenates of rat paws treated with intra-plantar carageenan (Guay *et al.*, 2004), as well as supernatants of *ex vivo* cultured human synovium (Robinson *et al.*, 1975a; Robinson *et al.*, 1975b) and monolayers of dissociated synovial cells taken from collagen-induced arthritic rats, but not non-arthritic rats (Robinson *et al.*, 1975a; Robinson *et al.*, 1975b).

However, of notable interest in this study, is the presence of PGE_2 in samples from naïve joints. There are two COX isoforms; the relative amount of each isoform expressed in individual tissues varies and is modulated under pathological conditions. It is therefore possible that the PGE_2 detected in normal joints was present prior to the needles being inserted, however, it is also possible that arachidonic acid from the joint tissue was metabolised to PGE_2 as a result of the mechanical stimulation caused by the needles (Ferreira & Vane, 1967). However, PGE_2 also plays a role in bone homeostasis (Kawaguchi *et al.*, 1995); so it may be that the levels detected in normal joints may represent endogenous prostanoids. This could be confirmed by analysing the PGE_2 content of whole homogenised joints which have not undergone an injection or other procedure. If PGE_2 is present in these samples, then it is likely that the PGE_2 detected in the present study was in the joint prior to the needle insertion.

Finally, the total number of inflammatory cells present in the joint perfusate samples was investigated. Not surprisingly it was found that FCA-injected joints contained higher levels of inflammatory cells than normal rat knee joints, as previously reported (Santos & Tipping, 1994). However, of particular interest are the inflammatory cell counts from contralateral, non-injected limbs. Contralateral effects arising from a unilateral insult is a well documented phenomenon. In general, the changes in behaviour, magnitude of biochemical fluctuations or histopathological lesions in the contralateral joint are less than those observed on the injected joint (for review see Shenker *et al.*, 2003). Total

inflammatory cell count data from this study are in agreement with this finding, and although the lower dose of FCA used here does not elicit behavioural signs of inflammation or hypersensitivity in the contralateral joint, there is evidence of infiltration of inflammatory cells. Furthermore, the higher dose of FCA induced contralateral invasion of inflammatory cells to similar levels seen in the ipsilateral side of low dose FCA injected animals, indicating the onset of contralateral spread with higher FCA levels (Donaldson *et al.*, 1993; Shenker *et al.*, 2003).

In summary, a novel method for sampling synovial fluid content via perfusing the joint cavity has been devised and used successfully for obtaining samples for subsequent analysis of cytokine and prostanoid levels during adjuvant-induced arthritis. This method has the advantage of enabling the contents of synovial fluid to be investigated alone, without the contamination of the surrounding tissue. We have also shown that it can be employed to elucidate the temporal expression pattern of PGE_2 during the development and progression of rat-adjuvant arthritis and revealed its value in measuring cellular components of inflammation.

Chapter 5 – The effect of adjuvant-induced arthritis on the expression of inflammatory cells and cytokines in the synovial cavity

5.1 INTRODUCTION

The complex actions and interactions of inflammatory proteins that drive the immune response in the joint are still not fully understood, although it is known that cytokines, in particular, TNFα, IL1β and IL6, are important in the development and maintenance of RA (Brennan *et al.*, 1992; Choy & Panayi, 2001; Feldmann *et al.*, 1990). The expression levels of these molecules are elevated in the synovium of RA patients (Chikanza *et al.*, 1995a; Eastgate *et al.*, 1988; Houssiau, 1995; Houssiau *et al.*, 1988; Ozaki *et al.*, 2001; Saxne *et al.*, 1988). TNFα and IL6 are involved in inflammation, differentiation, proliferation of T and B cells and bone resorption (Campbell *et al.*, 2003; Ishihara & Hirano, 2002). IL1β is involved during the induction of inflammation, modification of the immune response and activation of osteoclasts (Dayer, 2003; Seckinger *et al.*, 1990).

Synovial biopsies from knee joints of RA patients analysed for cytokine immunolocalisation or mRNA levels identified a variable pattern (Alsalameh *et al.*, 1999; Barrera *et al.*, 2001; Deleuran, 1996; Dolhain *et al.*, 1998; Kirkham *et al.*, 1999; Ulfgren *et al.*, 2000). Although TNFα was present at high levels in some RA patients, it was undetectable in approximately half of patients. This absence of TNFα in some patients and the fact that anti-TNFα therapy is not efficacious in all cases of RA suggests multiple pathways and factors are involved. Moreover, the cytokine network may vary at different stages of the disease; therefore repeated sampling in large groups of RA patients is needed to shed more light on this issue. However, in patients with active RA, synovial fluid levels of IL6 have been reported to be between 160 and 660 pg ml^{-1} (Matsumoto *et al.*, 2006; McNiff *et al.*, 1995). Considerably higher levels (nearly 3000 pg ml^{-1}) have been reported in synovial fluid of patients with juvenile idiopathic arthritis (JIA; de Jager *et al.*, 2006). Several studies have investigated the levels of IL1β in the synovial fluid of RA patients; levels were approximately 360 pg ml^{-1} (McNiff *et al*, 1995), whereas they were reportedly only 15 pg ml^{-1} in patients with JIA (de Jager *et al.*, 2006).

Finally, TNFα levels have in patients with active RA and JIA ranged from 13 to 562 pg ml^{-1} (McNiff *et al.*, 1995; de Jager *et al.*, 2006).

Moreover, changes to the levels of proinflammatory cytokines in the blood are also seen n patients with inflammatory arthritis. De Jager *et al.* (2006) report that levels of IL1β, IL6 and TNFα in normal, healthy controls were 1.2, 0 and 1.1 pg ml^{-1} respectively. In contrast, serum levels of these proteins in arthritic patients have been reported to be 81-100, 440- 572, 6-1200 pg ml^{-1} (Danis *et al.*, 1992; de Jager *et al.*, 2006; Syesson *et al.*, 2002).

There is a need for new treatments, perhaps based on the antagonism of a combination of several inflammatory mediators. However, RA progresses over the course of years, making studying the effects of the disease course on cytokine expression difficult. Animal models of RA are widely used to gain insights into the pathogenic mechanisms of inflammation, processes of autoimmunity and during the preclinical development of new therapeutic agents. Rodent models of RA avoid these limitations, since the disease progresses over a period of a few weeks and serial sampling is more viable. However, none of the rodent models exactly mimic human RA.

Glucocorticoids, such as prednisolone, despite having a number of side effects, are potent and commonly used anti-inflammatory agents used to treat human RA. They are known to down-regulate pro-inflammatory cytokine production such as IL1β and TNFα normally produced by macrophages and monocytes (Kunicka *et al.*, 1993). In the present study, prednisolone was used to investigate the effect of steroid treatment on cytokine production in the arthritic rat knee joint.

The primary aim of this study was to characterise the dynamics of key pro- and anti-inflammatory cytokine expression in the knee joints of rats with adjuvant induced arthritis and compare these to behavioural measures of swelling and mechanical hypersensitivity. Secondly,

the effect of the steroid prednisolone on the cytokine content of arthritic rat knee joints was investigated.

5.2 METHODS

5.2.1 STUDY DESIGN

Normal joint and blood cytokine levels and cell counts

The body weights, joint diameters and weight distribution between the hind limbs were assessed prior to the perfusion of untreated joints (n=11). Samples were immediately frozen and stored at -20° C, and later assayed for multiple cytokines using the Luminex assay. Blood plasma samples were taken after perfusions of the knee joints were complete. In order to determine the total inflammatory cell count of perfusate samples, 50 μl was removed from the joint perfusate sample prior to freezing and transferred to a new centrifuge tube. A total inflammatory cell count was conducted.

Effect of FCA on joint and blood cytokine levels and inflammatory cell counts

Body weights, joint diameters and weight distribution between the hind limbs were assessed in rats prior to induction of joint inflammation (day 0). FCA (150 μg) was injected into the left (ipsilateral) knee joint under transient halothane anaesthesia (n=100).

Animals were assessed for changes in body weight, weight distribution and joint diameter (ipsilateral and contralateral) prior to the perfusion of the joint. The ipsilateral and contralateral knee joints from eight FCA-injected animals were perfused 2, 5, 7, 10, 14, 16, 18, and 21 days post-FCA (n=8 per day, n=64 in total), and from four FCA-injected rats on days 1, 3, 4, 8, 9, 11, 12, 15 and 17 (n= 4 per day, n=36 in total). Samples were immediately frozen and stored at -20° C, and later assayed for multiple cytokines using the Luminex assay. Blood plasma samples were taken after perfusions of the knee joints were complete. In order to

determine the total inflammatory cell count of joint perfusate samples, 50 μl was removed from the perfusate sample prior to freezing and transferred to a new 1 ml centrifuge tube. A total inflammatory cell count was conducted.

Effect of prednisolone on FCA-evoked joint inflammation

Body weights, joint diameters and weight distribution between the hind limbs were assessed in rats prior to induction of joint inflammation (day 0). FCA (150 μg) was injected into the left (ipsilateral) knee joint under transient halothane anaesthesia (n=54). The knee joints from six FCA-injected animals were perfused 3, 7 and 10 (n=18 in total) days post-FCA. On day 13, all remaining arthritic rats (n=36) were injected (s.c.) with prednisolone (10 mg kg^{-1}); the ipsilateral and contralateral knee joints of six of these rats were perfused one hour later. On days 14 (n=30), 15 (n=24), 16 (n=18) and 17 (n=12) the protocol was repeated and the joints of six rats were perfused daily one hour after prednisolone treatment. On day 21 the knee joints of the final six rats were perfused. All samples were frozen and stored at -20° C, and later assayed for multiple cytokines using the Luminex assay.

5.3. RESULTS

5.3.1. NORMAL ANIMALS

Cytokine content of untreated rat knee joints

In general, cytokine levels in normal joint perfusate samples (n=10) were below the level of detection of the assay. However, some samples did contain detectable levels of some cytokines (see Table 5.1).

Cytokine	IL1α	IL1β	IL2	IL4	IL6	IL10	GM-CSF	IFNγ	TNFα
Concentration (pg ml^{-1})	0.9 ± 0.6	1.2 ± 1.2	0.2 ± 0.2	0.3 ± 0.3	3.2 ± 3.2	1.9 ± 1.3	0.2 ± 0.2	0.2 ± 0.2	0.3 ± 0.3
Number of samples	11	11	10	10	10	10	10	10	10
Number of samples with detectable levels	2	1	1	1	1	2	1	1	1

Table 5.1 The concentrations of each cytokine (in pg ml^{-1}), the total number of rats sampled and the number of joints that contained detectable levels of each analyte in normal (untreated) rat knee joints. Detection limit of the assay was 2 pg ml^{-1}: this data represents anything <2 =0 and anthing greater han 2 pg ml^{-1} averaged. Note: it was not the same samples that contained measurable concentrations of each cytokine.

Cytokine content of untreated rat plasma

Blood samples from normal rats showed negligible levels of eight of the nine cytokines measured, however levels of TNFα were 4.7 ± 4.7 pg ml^{-1} as a result of one sample containing 18.8 pg ml^{-1} TNFα (n=4; see Figure 5.1).

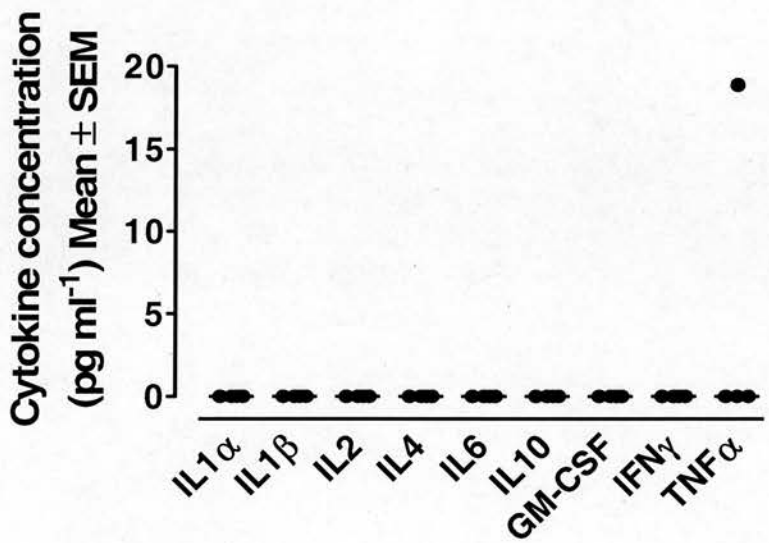


Figure 5.1 Levels of cytokines in blood samples from normal (untreated; n=11) rats. All cytokines assayed were below the detection threshold of the assay, except for TNFα which had a concentration of approximately 4 pg ml^{-1}.

5.3.2 EFFECT OF FCA ON LEVELS OF CYTOKINE EXPRESSION IN THE BLOOD AND JOINT CAVITIES

Behavioural assessments

FCA had no adverse effects on the animals' general health, as evidenced by the fact that animals continued to feed and gain weight normally throughout the study (see Figure 5.2a). On days 16, 18 and 21-post FCA, the body weights of the rats were significantly higher than day 0 ($P<0.05$, Kruskal Wallis).

Before induction of arthritis the ipsilateral and contralateral knee joint diameters were 9.7 ± 0.1 mm (n=64) and 9.8 ± 0.1 mm (n=64) respectively, these values did not differ significantly ($P>0.05$, Mann Whitney). Following FCA, the ipsilateral joint was significantly swollen (13.8 ± 0.4 mm on day 2 post-FCA; n=8) in comparison with the contralateral joint (9.7 ± 0.2 mm on day 2 post-FCA; n=8; $P<0.05$, Mann Whitney). The ipsilateral joint diameter remained significantly higher than that of the contralateral joint until the end of the study ($P<0.05$, Kruskal Wallis; see Figure 5.2b). No changes in contralateral joint diameters occurred in comparison to the basal values at any time ($P>0.05$, Kruskal Wallis).

I.art FCA evoked mechanical hypersensitivity of the ipsilateral joint, as measured by the incapacitance tester. Prior to induction of joint inflammation, the weights carried by the two hind limbs were 102.6 ± 3.1 g (n=64) and 104.3 ± 3.3 g (n=64). However, following administration of FCA the weights on the ipsilateral and contralateral hind limbs were 23.3 ± 2.7 g (n=8) and 132.9 ± 5.9 g (n=8) respectively; these were significantly different from each other ($P<0.05$, Kruskal Wallis). The weights on the two hind limbs remained significantly different from one another for the duration of the study ($P<0.05$, Kruskal Wallis; see Figure 5.2c). The associated ratio of the weight distribution between the hind limbs on day 0 was 0.99 ± 0.0 (n=64); two days after FCA the ratio was 0.19 ± 0.0 (n=8), this was significantly lower than the

basal measurement ($P<0.05$, Mann Whitney), and remained significantly reduced up to and including day 21, when the study was terminated ($P<0.05$, Kruskal Wallis; see Figure 5.2d).

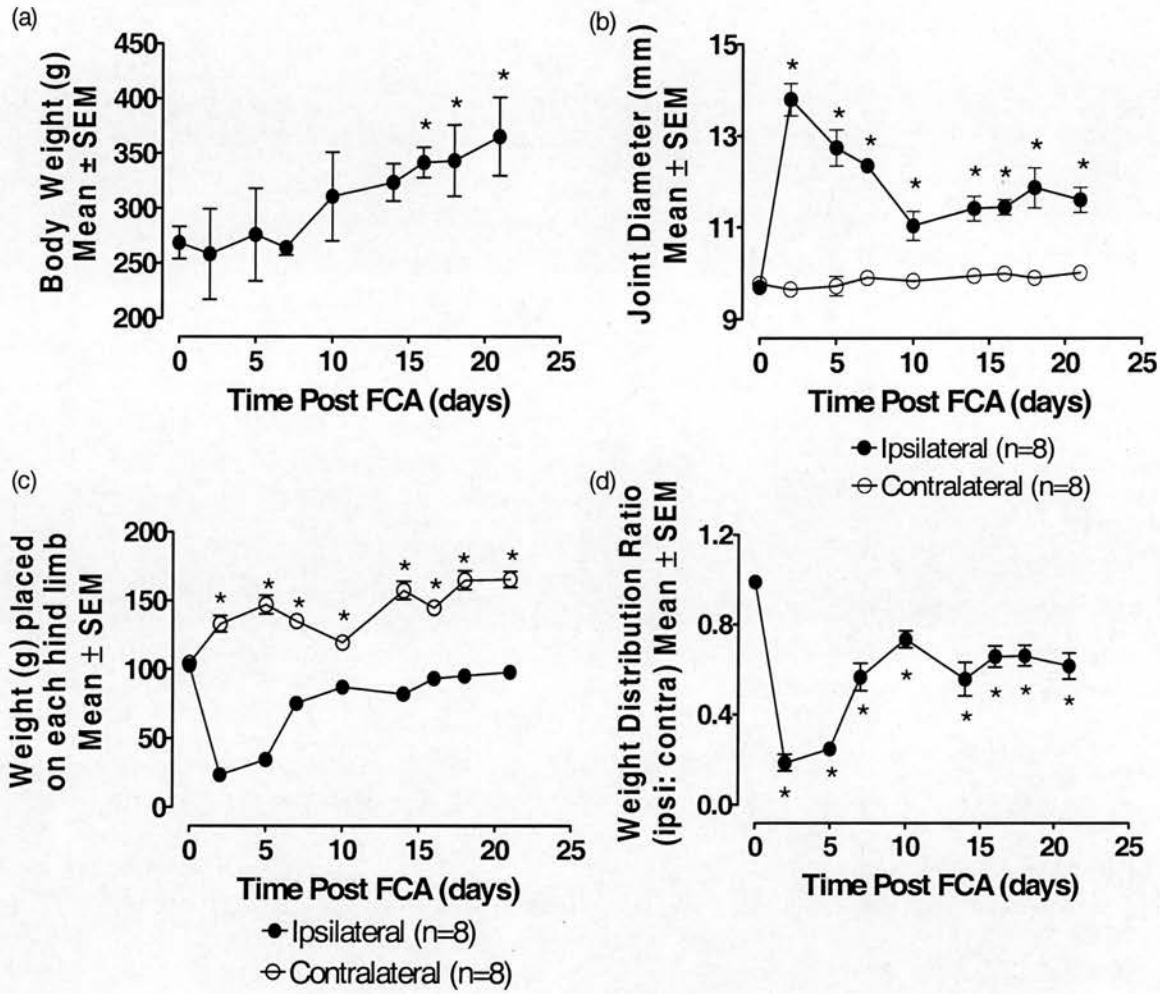


Figure 5.2 Measurements of (a) body weight, (b) knee joint diameter, (c) the weight placed on each hind limb and (d) the associated ratio of weight distribution between the ipsilateral and contralateral hind limbs of rats injected with FCA on day 0 (150 μl; n=8). The ipsilateral joints were significantly swollen and hypersensitive from day one post FCA until the end of the study on day 21 ($P<0.05$, Kruskal Wallis; represented by *).

Cytokine content of ipsilateral joints

Joint perfusates from rats injected with FCA (150 μl; on day 0) were analysed for multiple cytokine content using the Luminex Assay. IL1α, IL1β, IL2, IL4, IL6, IL10, GM-CSF,

IFNγ and TNFα concentrations of the samples were measured. Data are grouped into three day blocks, to reduce variations in temporal expression patterns between different rats.

Interleukin 1α

The concentration of IL1α in the ipsilateral (injected) joint was significantly elevated compared with values from normal joints in untreated rats on each test day until the end of the study on day 21 (n=9-21; $P<0.05$, Kruskal Wallis; see Figure 5.3a). The peak IL1α concentration occurred between days 16 and 18, when the level was 56.7 ± 19.2 pg ml^{-1} (n=17). See Figure 5.3b for the scatter of IL1α concentrations from ipsilateral joints. The proportion of IL1α-positive samples on particular days were compared with those of untreated joints (day 0) using the Fisher's Exact test (see Figure 5.3c). In each time group, the proportion of IL1α-positive joints was significantly greater than in normal joints ($P<0.05$, Fisher's Exact test).

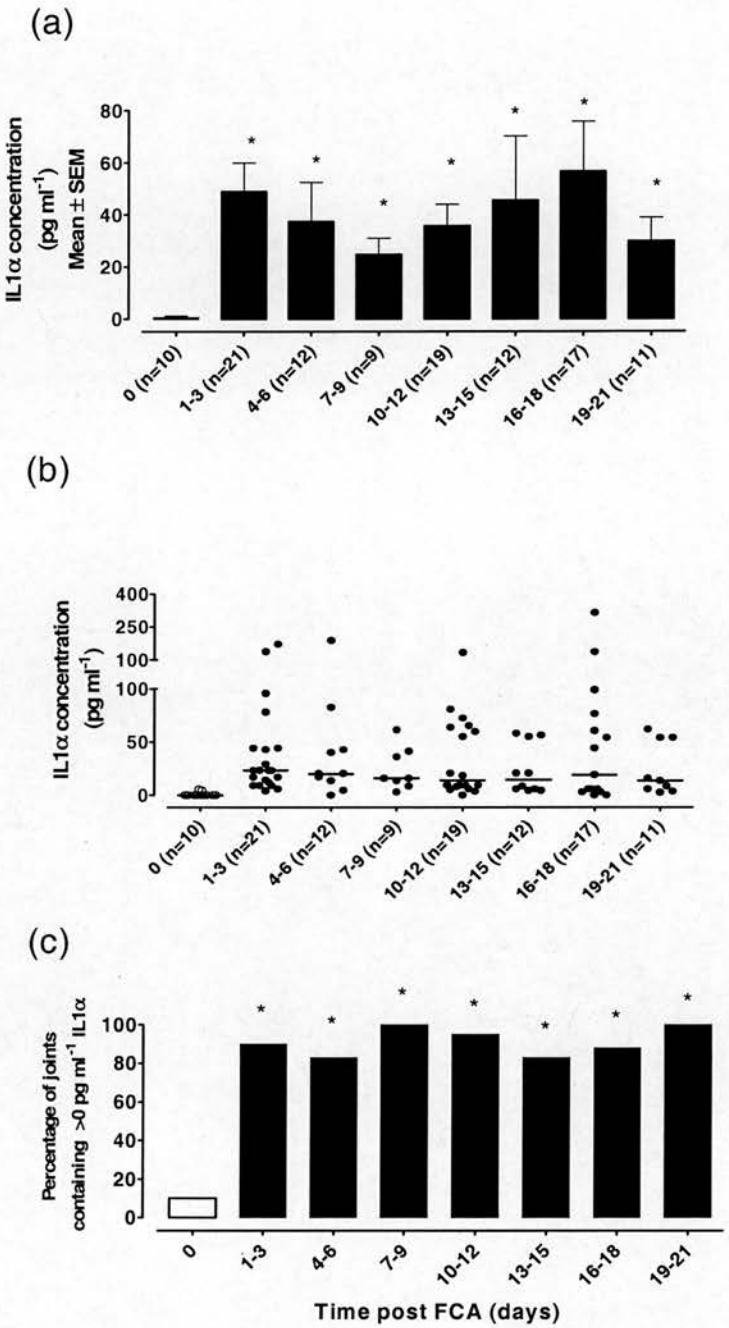


Figure 5.3 (a) Concentrations of IL1α in ipsilateral perfusate samples (n=10-21) and (b) the ranges of IL1α levels in perfusates from ipsilateral joints following FCA; the horizontal bar on the graph indicates the median concentration. (c) The percentage of ipsilateral knee joints that were positive for IL1α, *i.e.* they contained detectable levels of IL1α. Statistical significance ($P<0.05$) is represented by *, statistical analysis for figure (a) was carried out to compare concentrations on each day post FCA with levels in normal joints (day 0) using the Kruskal Wallis test. The Fisher's Exact test was used to analyse the differences in the proportion of samples that had detectable levels of the protein in (c).

Interleukin 1β

The level of IL1β in inflamed joints was significantly elevated in comparison with values from normal joints between days 1-3 (n=19), 4-6 (n=12) and 10-12 (n=19) post-FCA ($P<0.05$, Kruskal Wallis; see Figure 5.4a). The peak IL1β concentration (385.4 ± 203.9 pg ml^{-1}) occurred between days four and six. See Figure 5.4b for the scatter of IL1β concentrations from ipsilateral joints. The proportion of IL1β-positive samples on particular days were compared with those of untreated joints (day 0) using the Fisher's Exact test (see Figure 5.4c). In each time group, apart from between days 13-15 post-FCA, the proportion of IL1β-positive joints was significantly greater than in normal joints ($P<0.05$, Fisher's Exact test).

Interleukin 6

The concentration of IL6 in ipsilateral joint perfusates was not significantly different from normal joints at any time point post-FCA (n=9-20; $P>0.05$, Kruskal Wallis; see Figure 5.5a). See Figure 5.5b for the scatter of IL6 concentrations from ipsilateral joints. The proportion of IL6-positive samples on particular days were compared with those of untreated joints (day 0) using the Fisher's Exact test (see Figure 5.5c). In each time group, the proportion of IL6-positive joints was significantly greater than in normal joints ($P<0.05$, Fisher's Exact test).Between days 1-3 post-FCA, the proportion of IL6-positive joints was significantly higher than in normal joints ($P<0.05$, Fisher's Exact test).

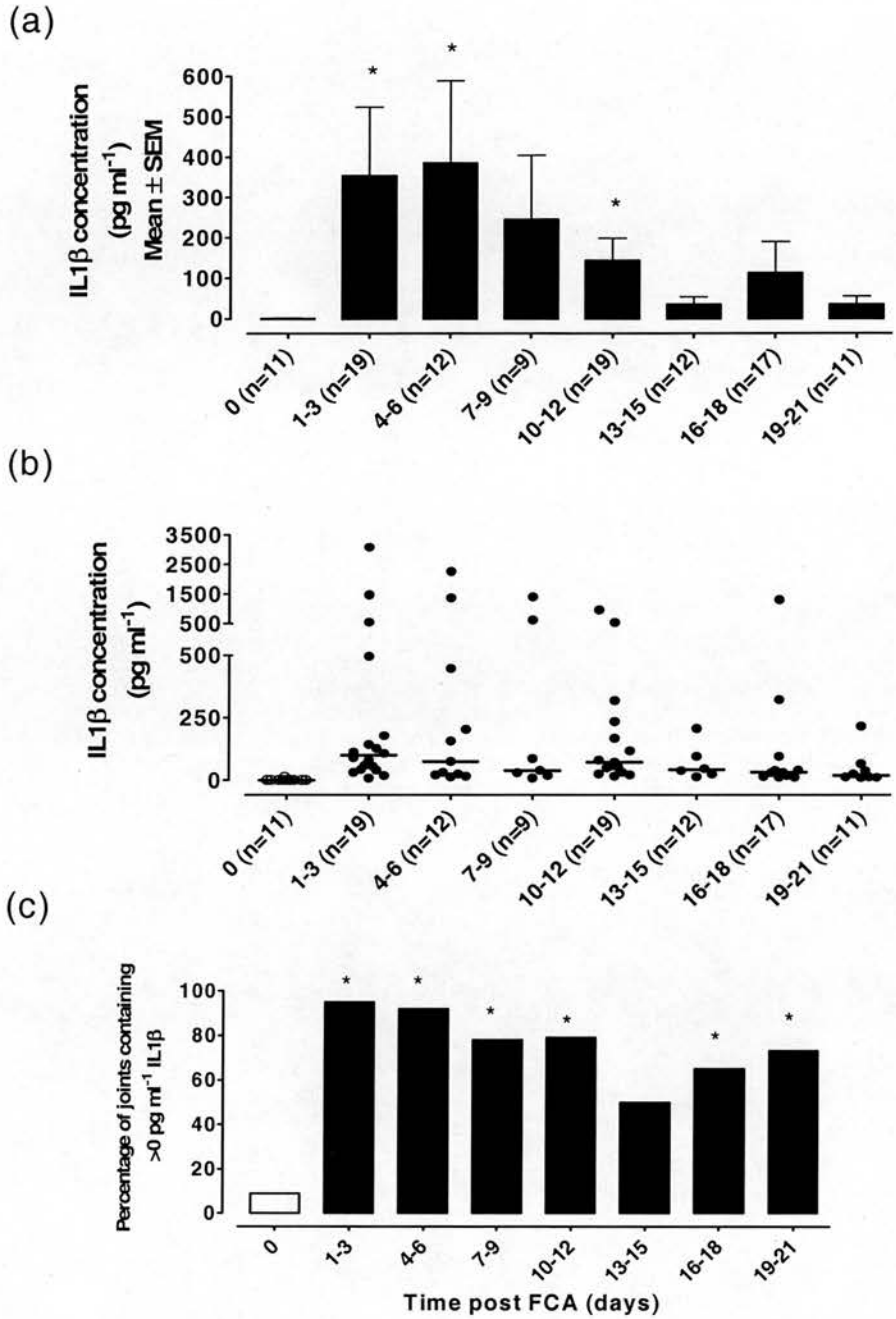


Figure 5.4 (a) Concentrations of IL1β in ipsilateral perfusate samples (n=10-21) and (b) ranges of IL1β levels in perfusates from ipsilateral joints following FCA; the horizontal bar on the graph indicates the median concentration. (c) The percentage of ipsilateral knee joints that were positive for IL1β, *i.e.* they contained detectable levels of IL1β. Statistical significance ($P<0.05$) is represented by *, statistical analysis for figure (a) was carried out to compare concentrations on each day post FCA with levels in normal joints (day 0) using the Kruskal Wallis test. The Fisher's Exact test was used to analyse the differences in the proportion of samples that had detectable levels of the protein in (c).

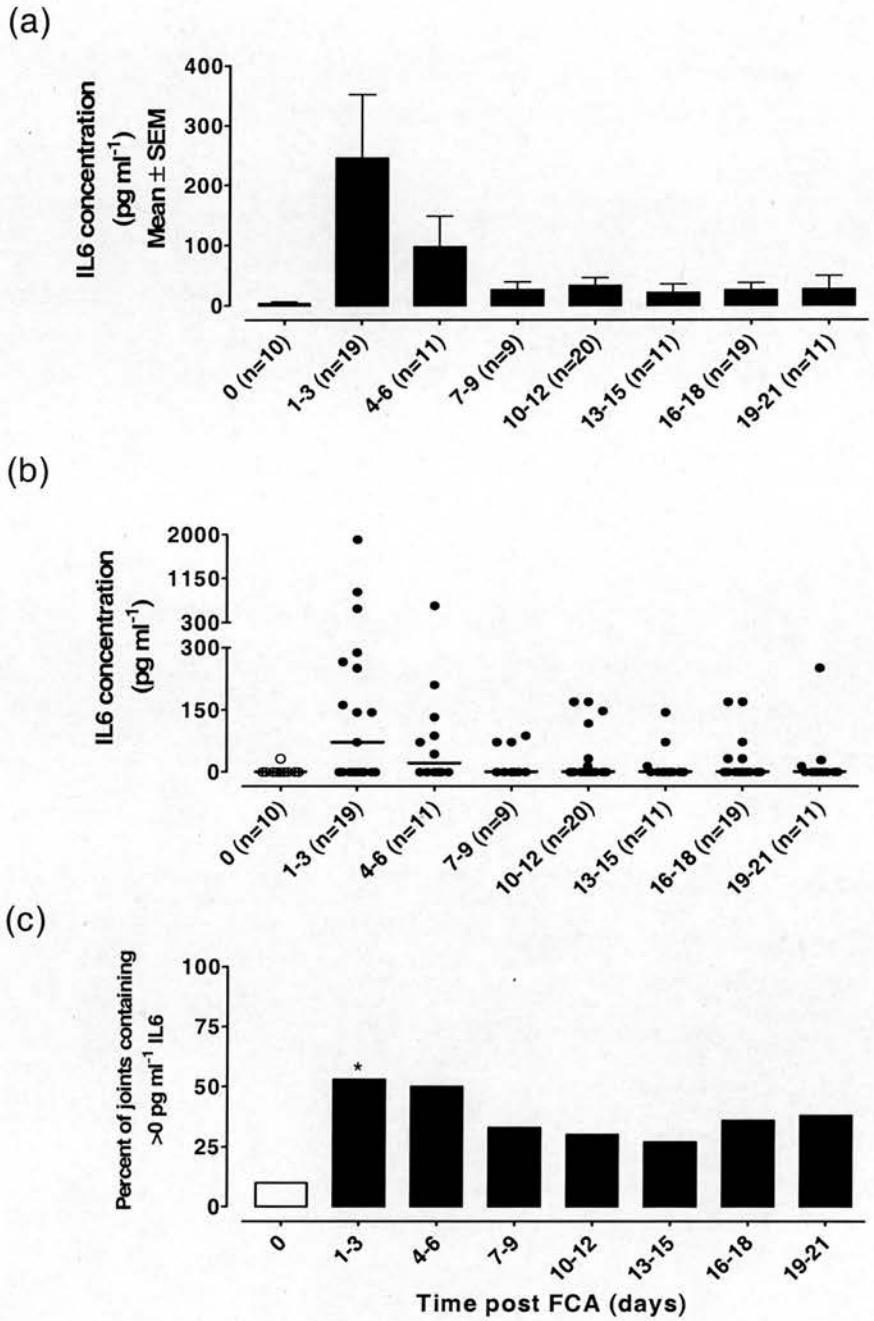


Figure 5.5 (a) Concentrations of IL6 in ipsilateral perfusate samples (n=8-20) and (b) the ranges of IL6 levels in perfusates from ipsilateral joints following FCA; the horizontal bar on the graph indicates the median concentration. (c) The percentage of ipsilateral knee joints that were positive for IL6, *i.e.* they contained detectable levels of IL6. Statistical significance ($P<0.05$) is represented by *, statistical analysis for figure (a) was carried out to compare concentrations on each day post FCA with levels in normal joints (day 0) using the Kruskal Wallis test. The Fisher's Exact test was used to analyse the differences in the proportion of samples that had detectable levels of the protein in (c).

Interleukin 10

The levels of IL10 in ipsilateral joint perfusates were not significantly different from those of normal joints at any time point post-FCA (n=9-21; $P>0.05$, Kruskal Wallis; see Figure 5.6a). See Figure 5.6b for the scatter of IL10 concentrations from ipsilateral joints. The proportion of IL10-positive samples on particular days were compared with those of untreated joints (day 0) using the Fisher's Exact test (see Figure 5.6c). Between 4 and 6 days post-FCA the proportion of IL10-positive joints was significantly higher than in normal joints ($P<0.05$, Fisher's Exact test).

Tumour Necrosis Factor α

The concentration of TNFα was significantly elevated in FCA-injected joints compared with normal joints between days 1 and 3 post-FCA (n=19; $P>0.05$, Kruskal Wallis; see Figure 5.7a). The peak TNFα concentration occurred between days 1-3 post-FCA, when the concentration was 14.9 ± 3.7 pg ml^{-1}. See Figure 5.7b for the scatter of TNFα concentrations from ipsilateral joints. The proportion of TNFα-positive samples on particular days were compared with those of untreated joints (day 0) using the Fisher's Exact test (see Figure 5.7c). Until day 15 post-FCA the proportion of TNFα-positive joints was significantly higher than in normal joints ($P<0.05$, Fisher's Exact test).

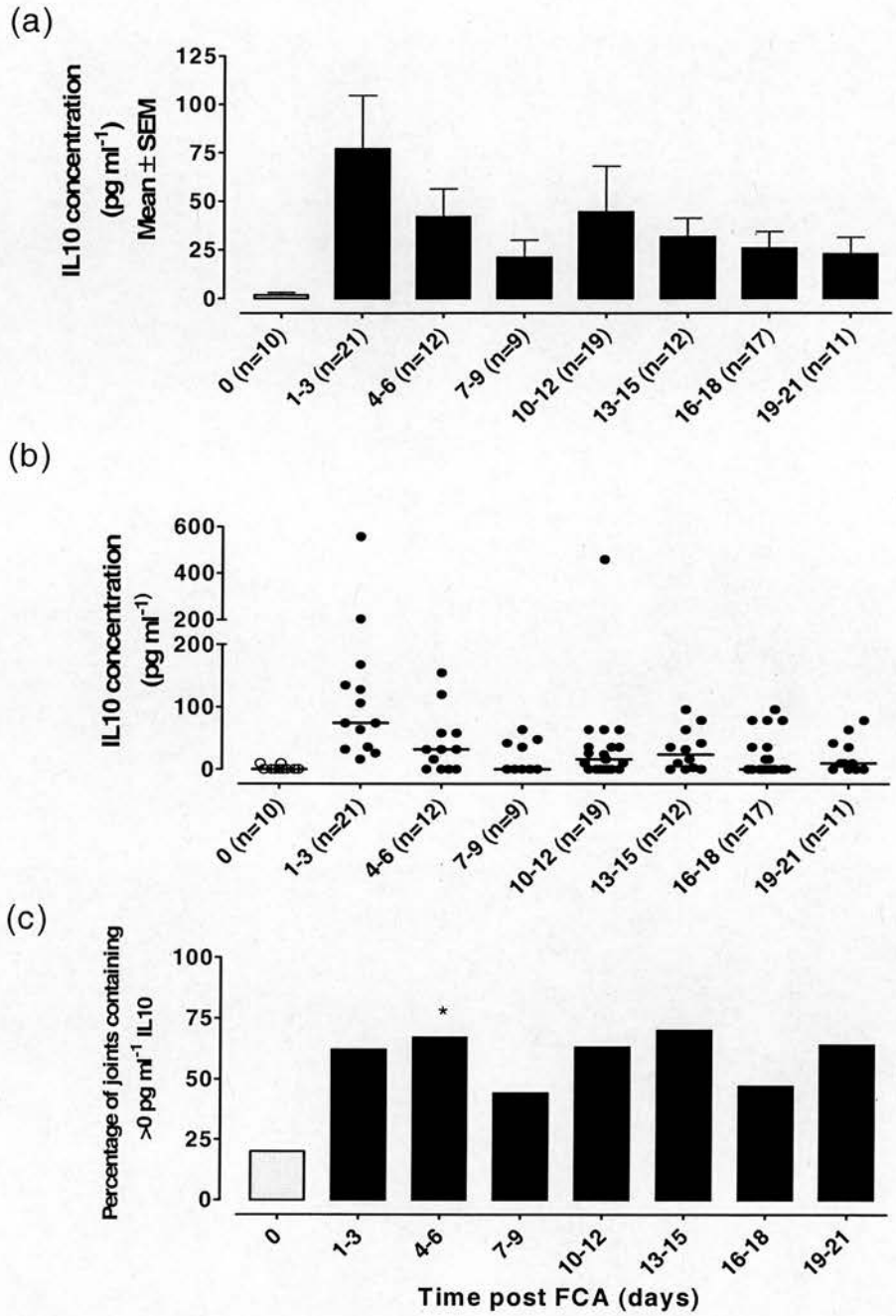


Figure 5.6 (a) Concentrations of IL10 in ipsilateral perfusate samples (n=8-21) and (b) ranges of IL10 in perfusates from ipsilateral joints following FCA; the horizontal bar on the graph indicates the median concentration. (c) The percentage of ipsilateral knee joints that were positive for IL10, *i.e.* they contained detectable levels of IL10. Statistical significance ($P<0.05$) is represented by *, statistical analysis for figure (a) was carried out to compare concentrations on each day post FCA with levels in normal joints (day 0) using the Kruskal Wallis test. The Fisher's Exact test was used to analyse the differences in the proportion of samples that had detectable levels of the protein in (c).

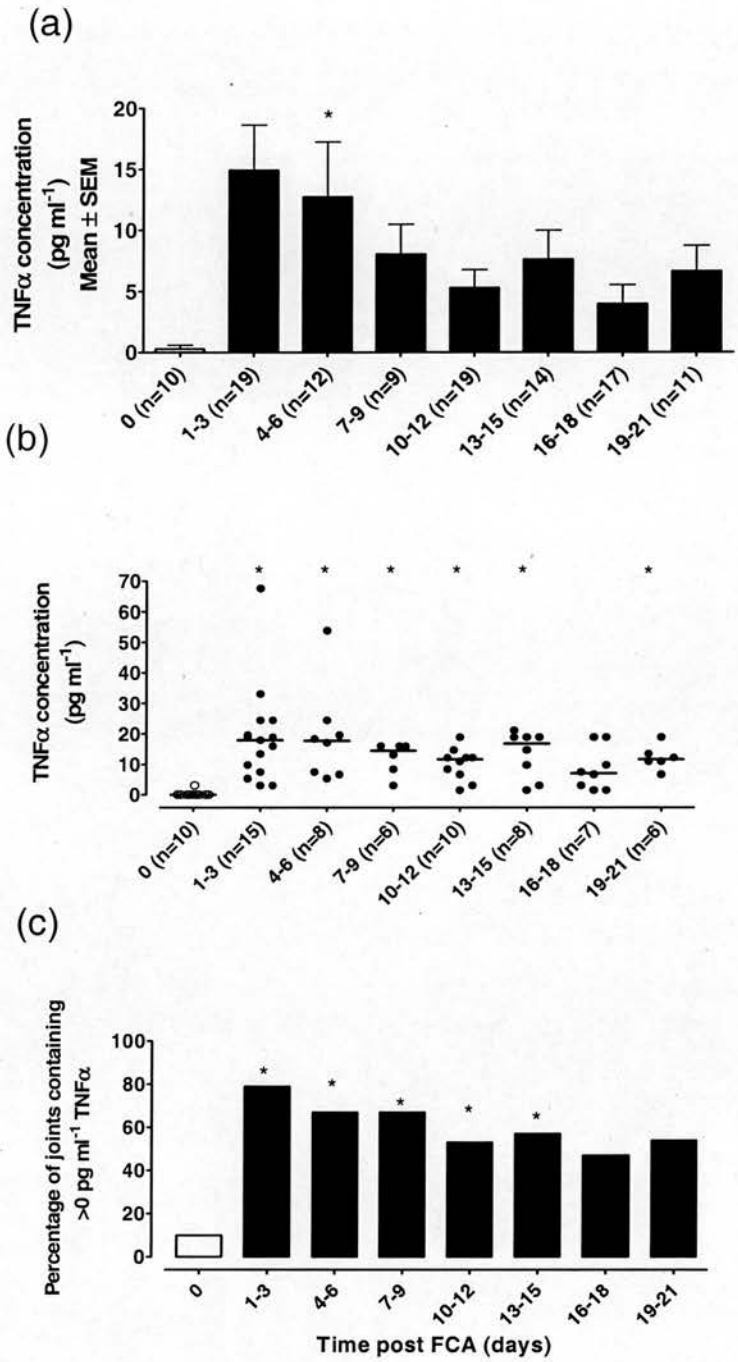


Figure 5.7 Concentrations of TNFα in (a) all perfusate samples (n=8-21) and (b) in perfusates from joints that were positive for TNFα following FCA (n=5-10); the horizontal bar on the graph indicates the median concentration. (c) The percentage of ipsilateral knee joints that were positive for TNFα, *i.e.* they contained detectable levels of TNFα. Statistical significance ($P<0.05$) is represented by *, statistical analysis for figure (a) was carried out to compare concentrations on each day post FCA with levels in normal joints (day 0) using the Kruskal Wallis test. The Fisher's Exact test was used to analyse the differences in the proportion of samples that had detectable levels of the protein in (c).

Interleukin 2 and 4, Granulocyte–Macrophage CSF and Interferon γ

Levels and spread of IL2, IL4, GM-CSF and IFNγ in FCA-injected and normal (untreated; day 0) knee joints of rats and the percentage of joints containing IL2, IL4, GM-CSF and IFNγ are shown in Table 5.2.

Cytokine content of contralateral joints

In addition to changes in cytokine expression in the FCA-injected joint, increases in levels of cytokines in contralateral joints also occurred (see Figure 5.9). However, at each time point the levels were lower in the contralateral side compared with the ipsilateral joint. Some increases proved to be significant, in comparison with levels in normal joints, see Figure 5.8 ($P<0.05$, Kruskal Wallis).

Correlation of behavioural assessments with cytokine contents of ipsilateral joint perfusates

In order to determine whether any of the cytokines assayed in the ipsilateral joint perfusion samples were significantly correlated with the behavioural measures (knee joint swelling and hypersensitivity) Spearman rank correlation factors were calculated (see Figure 5.9). IL1α, IL1β, IL4 and IL10 showed strong positive correlations with the ipsilateral knee joint diameter ($P<0.05$).

Correlations between levels of IL1α, IL1β, IL6 and TNFα in ipsilateral joint perfusates and the ratio of weight distribution on the hind limbs are shown in Figure 5.10. The correlations between these two parameters were significant ($P<0.05$, Spearman r).

	Day	IL2	IL4	GM-CSF	IFNγ
Levels in ipsilateral joint perfusates (Mean ±SEM; pg ml^{-1}) **All data**	**0**	0.2 ± 0.2 (n=10)	0.3 ± 0.3 (n=10)	0.1 ± 0.1 (n=10)	0.2 ± 0.2 (n=19)
	1 - 3	87.2 ± 47.3 (n=21)	10.2 ± 7.3 (n=15)	7.8 ± 3.1* (n=10)	1441.0 ± 890.0 (n=21)
	4 - 6	4.5 ± 2.5 (n=12)	20.0 ± 9.4 (n=13)	3.3 ± 1.2 (n=5)	728.5 ± 728.3 (n=12)
	7 - 9	21.9 ± 9.9 (n=9)	2.1 ± 1.6 (n=6)	2.5 ± 0.9 (n=4)	70.2 ± 66.5 (n=9)
	10 - 12	16.9 ± 5.9 (n=19)	4.8 ± 3.7 (n=9)	1.9 ± 0.5 (n=8)	1573.0 ± 1090.0 (n=19)
	13 - 15	8.2 ± 2.6 (n=12)	1.0 ± 1.0 (n=10)	1.5 ± 0.9 (n=4)	11.2 ± 4.8 (n=12)
	16 - 18	42.6 ± 22.4 (n=19)	33.8 ± 33.8 (n=5)	2.4 ± 1.1 (n=7)	2609.0 ± 1857.0 (n=17)
	19 - 21	8.7 ± 4.0 (n=11)	0.2 ± 0.2 (n=13)	2.0 ± 0.4 (n=7)	3754.0 ± 2570.0 (n=11)
Range of levels in ipsilateral joint perfusates (pg ml^{-1})	**0**	0 – 1.63 (n=10)	0 – 2.57 (n=10)	0 – 1.47 (n=10)	0 – 1.7 (n=10)
	1 - 3	0 – 732 (n=21)	0 – 107.7 (n=15)	0 – 31.7 (n=10)	0 – 12558 (n=21)
	4 - 6	0 – 24 (n=12)	0 – 107.7 (n=13)	0 – 7.1 (n=5)	0 – 8740 (n=12)
	7 - 9	0 – 96 (n=9)	0 – 9.95 (n=6)	0 – 4.03 (n=4)	0 – 601.3 (n=12)
	10 - 12	0 – 96 (n=19)	0 – 33.17 (n=9)	0 – 3.01 (n=8)	0 – 17298 (n=19)
	13 - 15	0 – 19 (n=12)	0 – 9.95 (n=10)	0 – 3.0 (n=4)	0 – 35.4 (n=12)
	16 - 18	0 – 434 (n=19)	0 – 169.2 (n=5)	0 – 8.0 (n=7)	0 – 31539 (n=17)
	19 - 21	0 – 34.3 (n=11)	0 – 2.58 (n=13)	0 – 3 (n=7)	0 – 24600 (n=11)
Percentage of joints containing 0 pg ml^{-1}	**0**	10	10	10	10
	1 - 3	57*	20	80*	52*
	4 - 6	25	46	80*	17
	7 - 9	78*	29	75*	22
	10 - 12	53*	22	75*	37
	13 - 15	50	10	50	33
	16 - 18	74*	20	57	53*
	19 - 21	45	8	86*	36

Table 5.2 Levels of IL2, IL4, GM-CSF and IFNγ in ipsilateral joints of normal (untreated; day 0; grey bars; n=10-21) and FCA-injected rats (150 μg). The ranges of concentrations measured in joint perfusates and the percentage of joints that contained >0 pg ml^{-1} for each of the latter are included. Statistical significance (P<0.05) is represent by *.

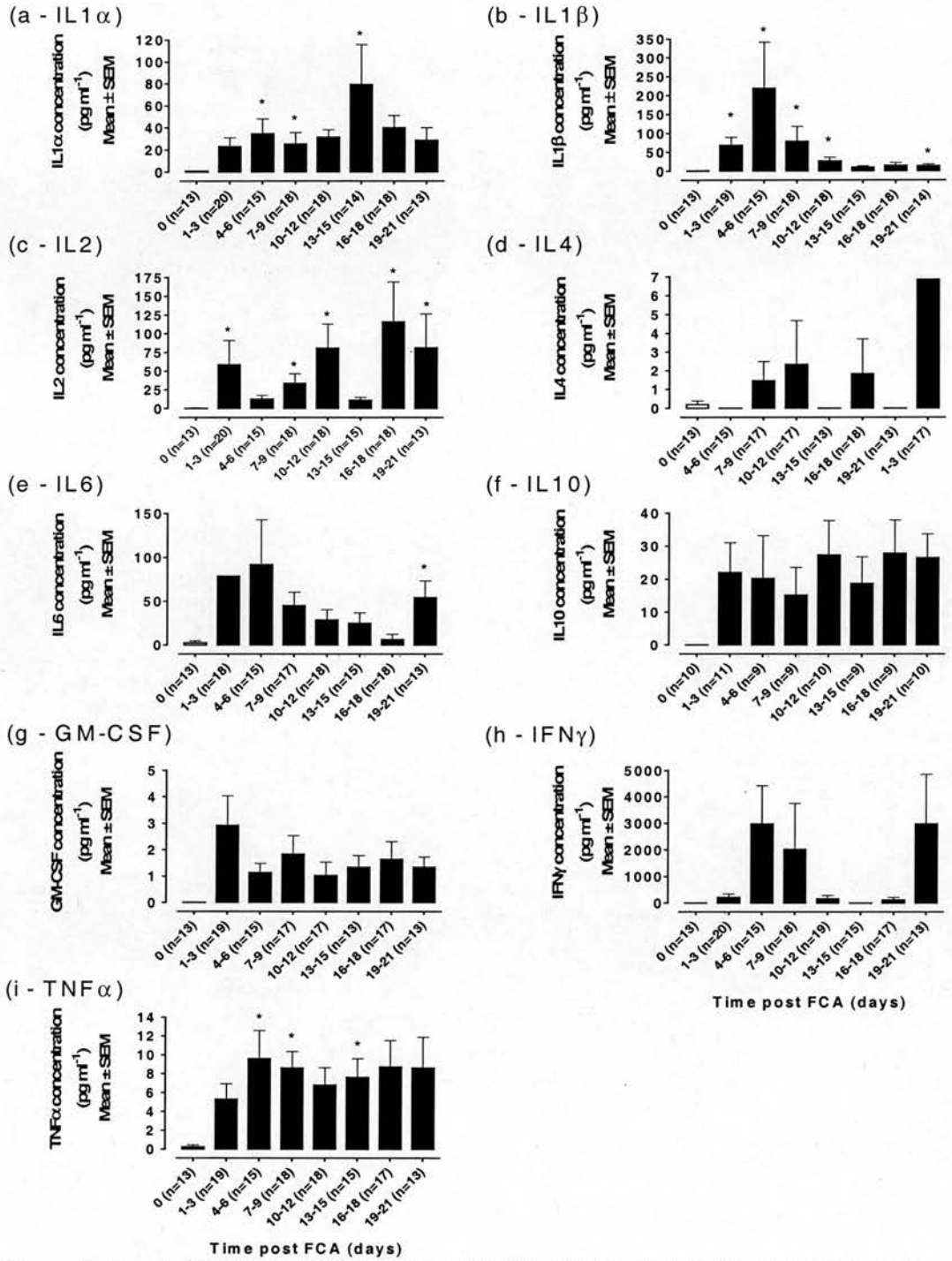


Figure 5.8 Levels of (a) IL1α, (b) IL1β, (c) IL2, (d) IL4, (e) IL6, (f) IL10, (g) GM-CSF, (h) IFNγ and (i) TNFα in joint perfusates from contralateral (uninjected; n= 9-20) rat knee joints following FCA (150 μl) injection into the ipsilateral joint on day 0. Statistical analysis was carried out to compare the concentration in each time block with the levels in normal joints using the Kruskal Wallis test; statistical significance (P<0.05) is represented by *.

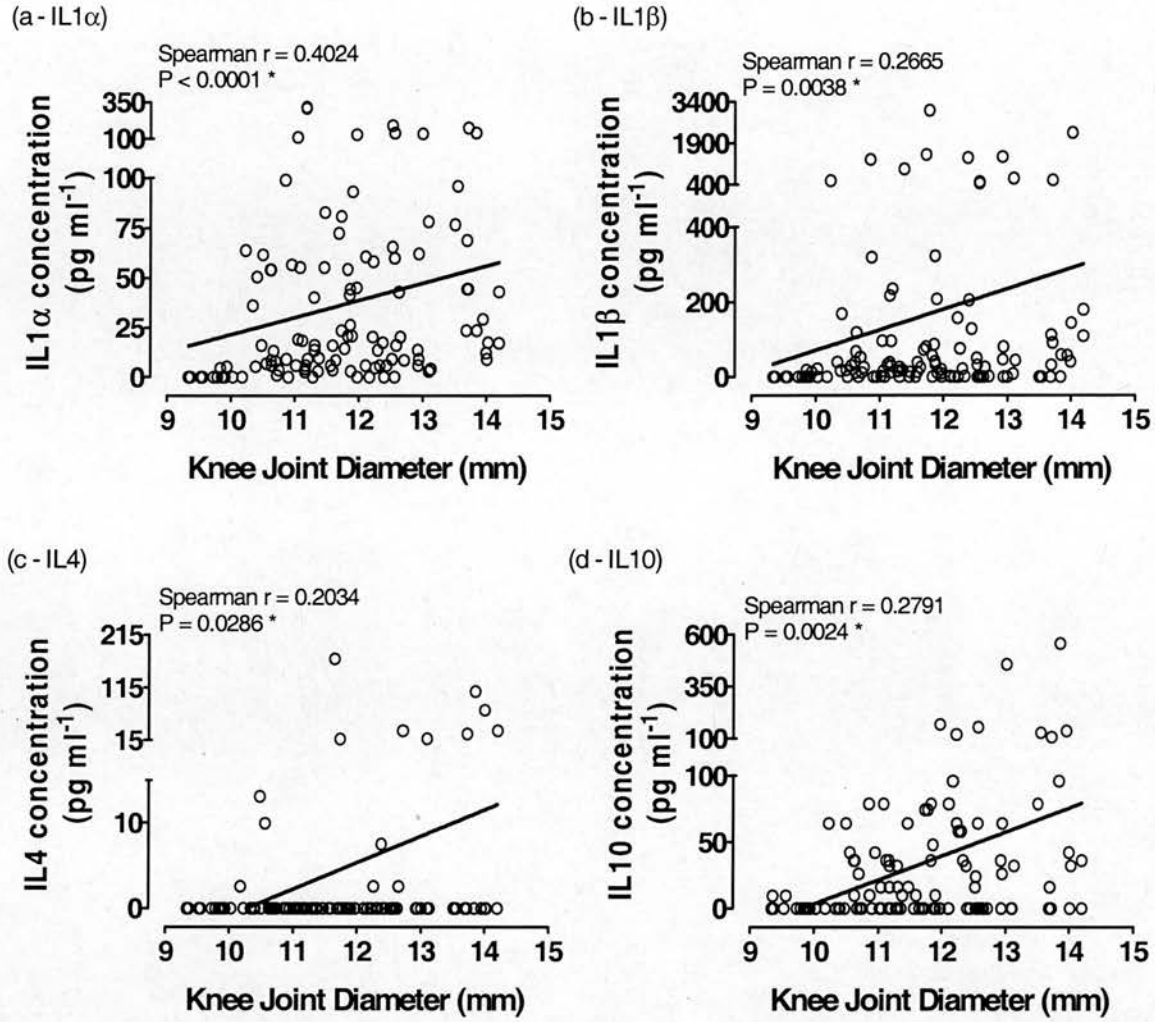


Figure 5.9 Correlation plots for ipsilateral joint diameter against the concentration of (a) IL1α, (b) IL1β, (c) IL4 and (d) IL10 in ipsilateral joint perfusates. Figures include the linear regression line and the values for Spearman r correlation factor, and the associated *P* value.

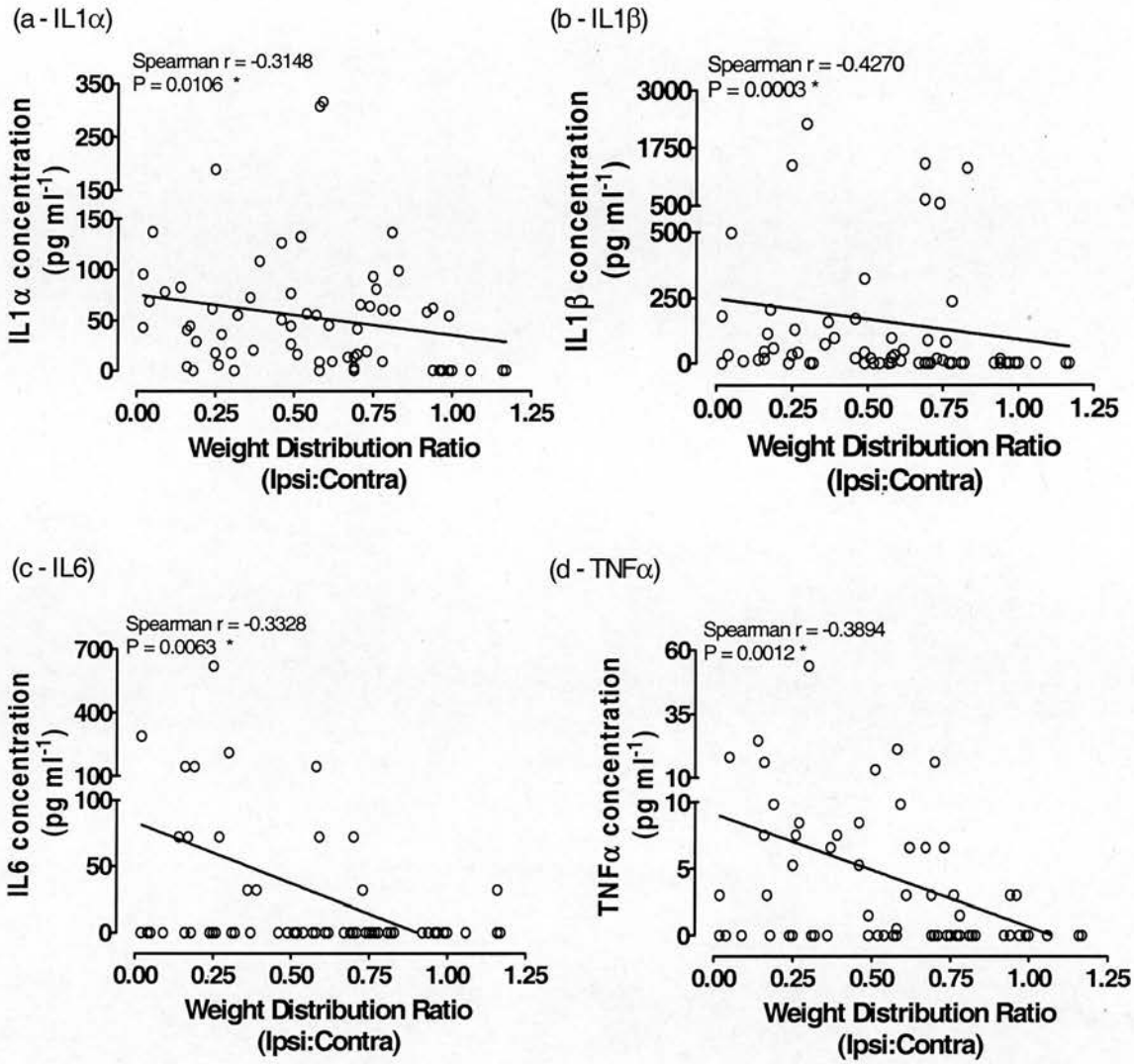


Figure 5.10 Correlation plots for the ratio of weight distribution between the hind limbs and the ipsilateral joint cytokine concentrations of (a) IL1α, (b) IL1β, (c) IL6 and (d) TNFα. Significant negative correlations occurred ($P<0.05$). Graphs include the linear regression plot and the values for Spearman r correlation factor, and the associated P value.

Cytokine content of blood samples

Induction of arthritis caused cytokine levels in blood plasma to increase in comparison with levels in blood from normal animals, however, none of the increases were statistically significant ($P>0.05$, Kruskal Wallis; see Figure 5.11).

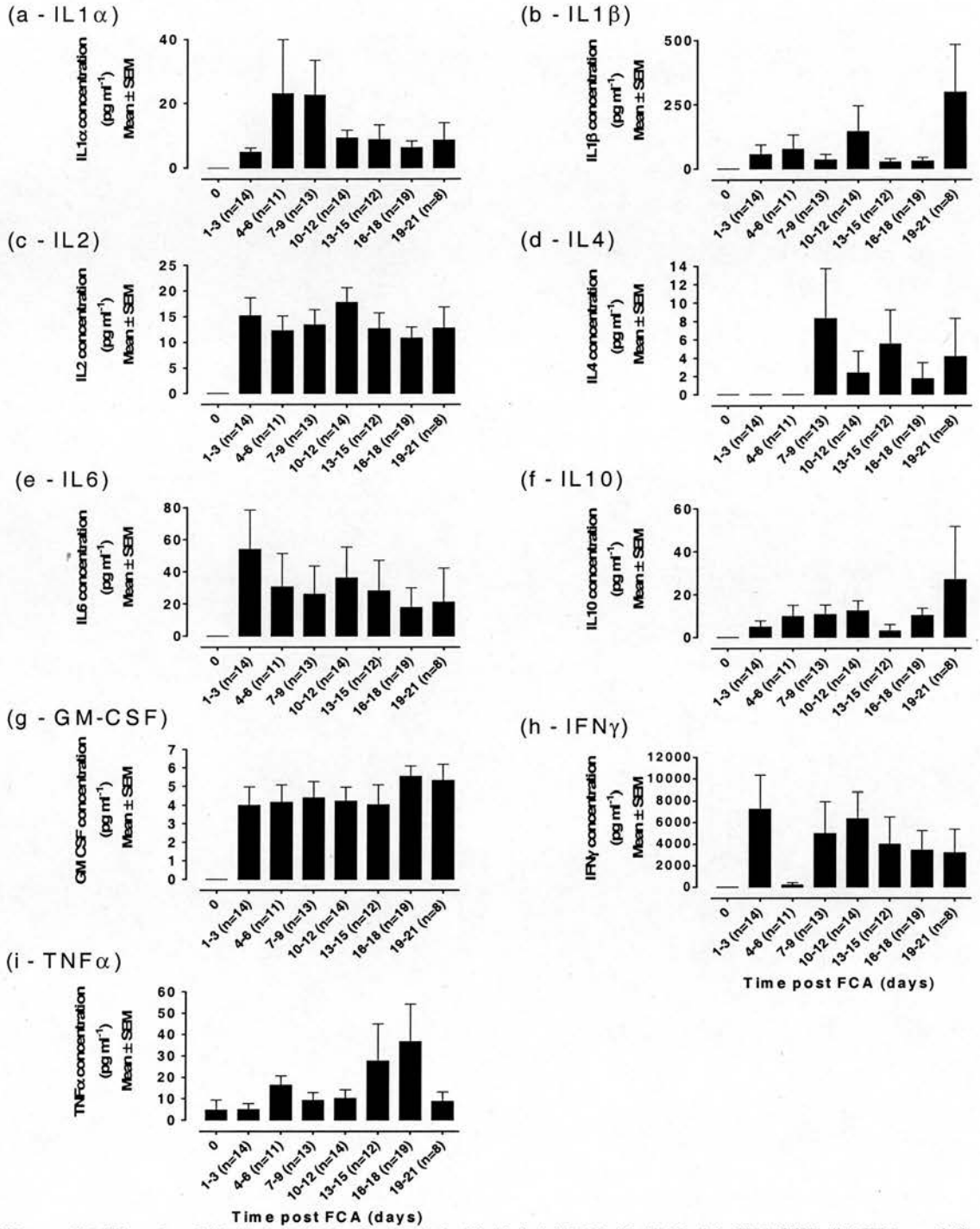


Figure 5.11 Levels of (a) IL1α, (b) IL1β, (c) IL2, (d) IL4, (e) IL6, (f) IL10, (g) GM-CSF, (h) IFNγ and (i) TNFα in blood plasma samples from rats injected with FCA (150 μg; on day 0; n= 8-19). Statistical analysis was carried out to compare the concentration in each time block with the levels in normal joints (day 0) using the Kruskal Wallis test; statistical significance (P<0.05) is represented by *.

Correlation of behavioural assessments with cytokine content of blood samples

No significant correlations between the cytokine content of blood samples and the joint swelling or hypersensitivity occurred in this study.

Inflammatory cell content of joints

Total inflammatory cell counts were carried out in joint perfusate samples from ipsilateral and contralateral knee joints of normal and FCA-injected rats (n=3 on each day). Increased numbers of inflammatory cells were counted on days 7, 10 and 21 in ipsilateral joints, but only on day 21 in contralateral joint perfusate samples. However, none of the increases were significant in comparison with samples from normal animals ($P>0.05$, Kruskal Wallis; see Figure 5.12).

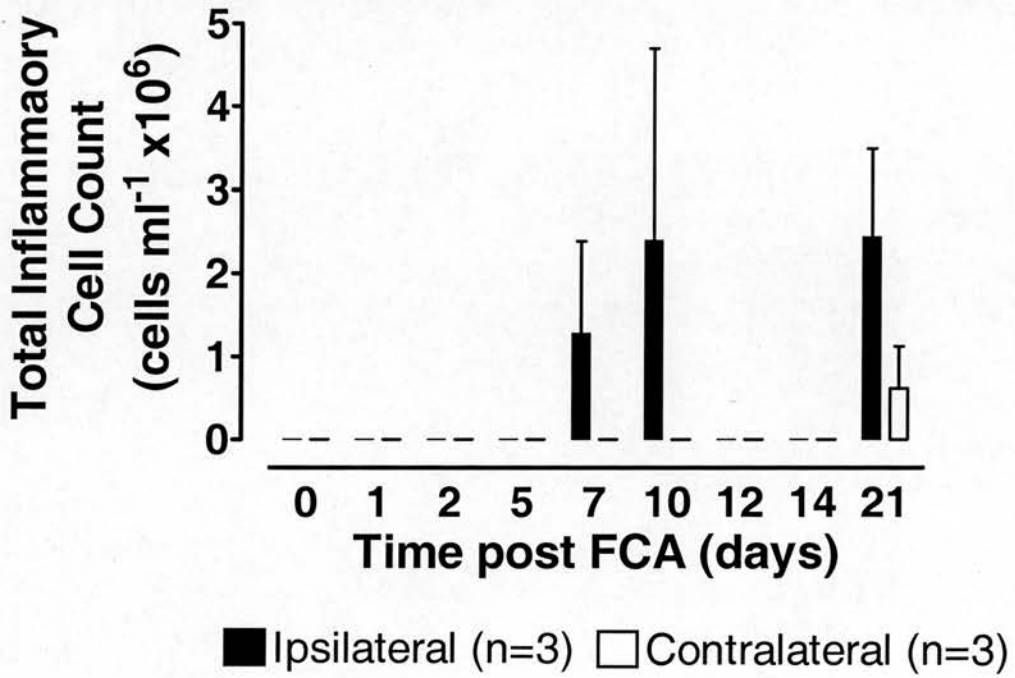


Figure 5.12 Total inflammatory cell counts in ipsilateral and contralateral joint perfusate samples from normal (day 0) and FCA-injected (150 μg; n=3) rat knee joints. No statistical differences in total cell counts were measured between FCA-injected and normal rat knee joints ($P>0.05$, Kruskal Wallis).

5.3.4 EFFECT OF PREDNISOLONE ON FCA-EVOKED INFLAMMATORY RESPONSE

Behavioural Assessments

FCA had no adverse effects on the rats' general health. Animals continued to feed and gain weight normally throughout the study. Data from days 0-9 are not shown, as no differences were seen between the values in this study and those in the FCA time course described in Section 5.3.2 ($P>0.05$, two-way ANOVA). No significant differences in body weight were measured between days 10 and 17 during drug treatment ($P>0.05$, Kruskal Wallis; see Figure 5.16a).

Ten days after FCA the ipsilateral knee joint was significantly swollen (12.3 ± 0.5 mm; n=6) in comparison with the contralateral joint (9.9 ± 0.0 mm; n=6; $P<0.05$, Mann Whitney). Prednisolone was administered on days 13 – 17 once daily (10 mg kg-1; s.c.). During prednisolone treatment swelling of the ipsilateral joint decreased, and by day 14 was no longer significantly swollen (11.3 ± 0.2 mm; n=6) compared with the contralateral joint (9.9 ± 0.1 mm; n=6; $P>0.05$, Kruskal Wallis; see Figure 5.16b).

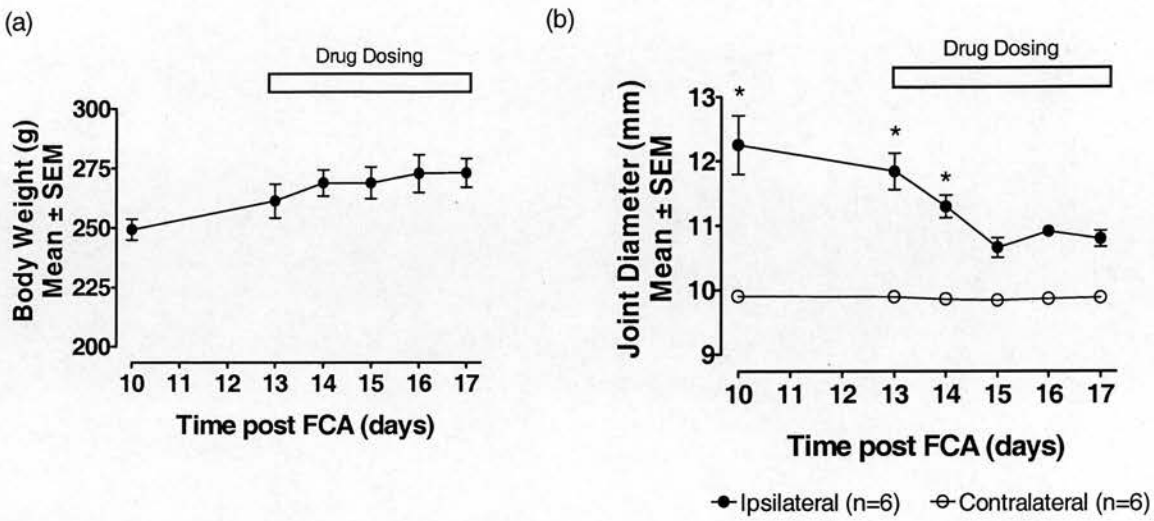


Figure 5.16 The (a) body weights and (b) knee joint diameters of the ipsilateral and contralateral limbs of rats injected with FCA on day 0. The joints were significantly swollen on day 10, prior to prednisolone administration between days 13 and 17 post-FCA. From day 14 onwards, the ipsilateral joint was no longer significantly swollen in comparison with the contralateral knee joint (n=6; $P<0.05$, Kruskal Wallis). Statistical significance ($P<0.05$) is represented by *.

Cytokine Content of joints - Ipsilateral

Joint perfusates from rats injected with FCA on day 0 were analysed for cytokine content using the Luminex Assay. Concentrations of IL1α, IL1β, IL2, IL4, IL6, IL10, GM-CSF, IFNγ and TNFα were measured. Data from day 10 until day 17 are shown, as data from days 0-9 showed no significant differences from the FCA study above (Section 5.3.2; $P>0.05$, two-way ANOVA). Prednisolone (10 mg kg^{-1}) was administered daily (s.c.) between days 13 and 17. Ipsilateral and contralateral joints were perfused approximately one hour after prednisolone injection.

Interleukin 1α

No significant differences were observed between levels of IL1α on day 10 (n=6; 21.3 ± 2.9 pg ml^{-1}; pre-prednisolone treatment) and any day during the drug-treatment regime in inflamed joints ($P>0.05$, Kruskal Wallis; see Figure 5.17a). Further data analysis was carried

out, in which all values below the level of detection of the assay (i.e. IL1α-negative joints) were removed from the data set to determine the effect of prednisolone treatment on the proportion of joints expressing IL1α protein. The IL1α content of IL1α-positive FCA-injected joints was not significantly different during prednisolone administration in comparison with the day 10, pre-treatment value (n=2-4; $P>0.05$, Kruskal Wallis; see Figure 5.17b). There were between one and four IL1α-negative joints in each time group; the proportion of "responder" samples on particular days was compared with those of pre-prednisolone treated levels (day 10) using the Fisher's Exact test (see Figure 5.17c). On day 16 the proportion of IL1α-positive joints was significantly less than in non-drug treated joints ($P<0.05$, Fisher's Exact test).

Interleukin 1β

No significant differences were observed between levels of IL1β on day 10 (n=6; 27.3 ± 4.7 pg ml^{-1}; pre-prednisolone treatment) and any day during the drug-treatment regime in ipsilateral joints ($P>0.05$, Kruskal Wallis; see Figure 5.18a). The IL1β content of IL1β-positive FCA-injected joints was not significantly different during prednisolone administration in comparison with the day 10, pre-treatment value (n=5-6; $P>0.05$, Kruskal Wallis; see Figure 5.18b). There was only one IL1β-negative joint, on day 16. Prednisolone treatment had no effect on the proportion of IL1β-positive joints ($P>0.05$, Fisher's Exact test; see Figure 5.18c).

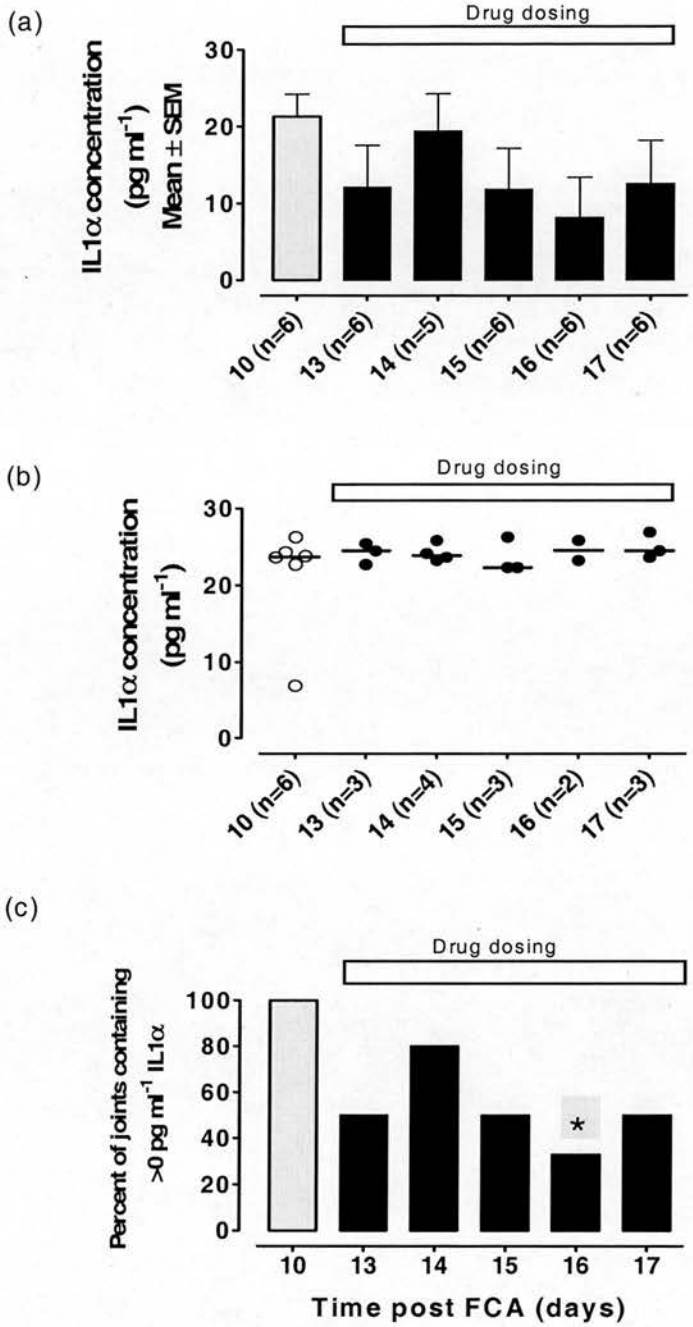


Figure 5.17 Concentrations of IL1α in (a) all joint perfusate samples and (b) in joints that were positive for IL1α content in pre-prednisolone treated rats (open symbols) and prednisolone-treated rats (closed symbols); the horizontal bar on the graph indicates the median concentration. (c) The percentage of FCA-injected (ipsilateral) knee joints that were positive for IL1α, *i.e.* contained detectable levels of this cytokine. Statistical significance ($P<0.05$) is represented by *. Statistical analysis for (a) and (b) were carried out to compare concentrations on each day post FCA with levels on day 10 using the Kruskal Wallis test. The Fisher's Exact test was used to analyse the differences in proportion of samples that had detectable levels of the protein in (c).

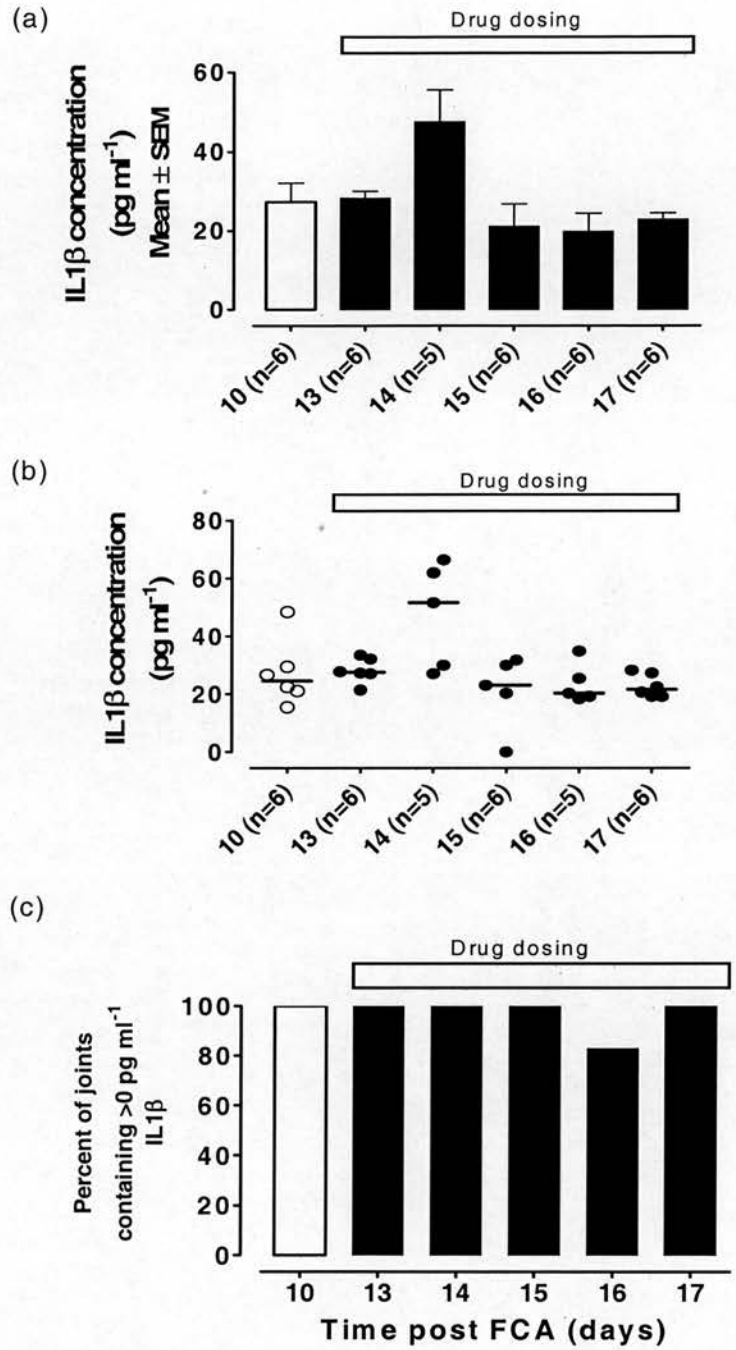


Figure 5.18 Levels of IL1β in (a) all joint perfusate samples and (b) in joints that were positive for IL1β content in pre-prednisolone treated rats (open symbols) and prednisolone-treated rats (closed symbols); the horizontal bar on the graph indicates the median concentration. (c) The percentage of FCA-injected (ipsilateral) knee joints that were positive for IL1β, *i.e.* contained detectable levels of this cytokine. Statistical significance ($P<0.05$) is represented by *. Statistical analysis for (a) and (b) were carried out to compare concentrations on each day post FCA with levels on day 10 using the Kruskal Wallis test. The Fisher's Exact test was used to analyse the differences in proportion of samples that had detectable levels of the protein in (c).

Interleukin 6

No significant differences between the concentration of IL6 before prednisolone treatment (day 10; n=6; 142.1 ± 67.3 pg ml^{-1}) and any day during the drug-treatment regime were seen (P>0.05, Kruskal Wallis; see Figure 5.19a). The IL6 content of IL6-positive FCA-injected joints was not significantly different during prednisolone administration in comparison with the day 10 levels (n=1-3; P>0.05, Kruskal Wallis; see Figure 5.19b). There were between three and five IL6-negative joints during prednisolone treatment. The Fisher's Exact test showed that prednisolone significantly reduced the proportion of IL6-positive joints on days 15 – 17 post FCA (following 3-5 days of prednisolone treatment; P>0.05, Fisher's Exact test; see Figure 5.19c).

Interleukin 10

No significant differences were observed between concentrations of IL10 in ipsilateral knee joints of FCA-injected animals on day 10 (58.0 ± 32.8 pg ml^{-1}; n=6; pre-prednisolone treatment) and any day during the drug-treatment regime (P>0.05, Kruskal Wallis; see Figure 5.20a). The IL10 content of IL10-positive FCA-injected joints was not significantly different during prednisolone administration in comparison with the day 10, pre-treatment value (P>0.05, Kruskal Wallis; see Figure 5.20b) and prednisolone had no significant effect on the proportion of IL10-positive joints (P>0.05, Fisher's Exact test), although there were between one and five IL10-negative joints during prednisolone treatment (see Figure 5.20c).

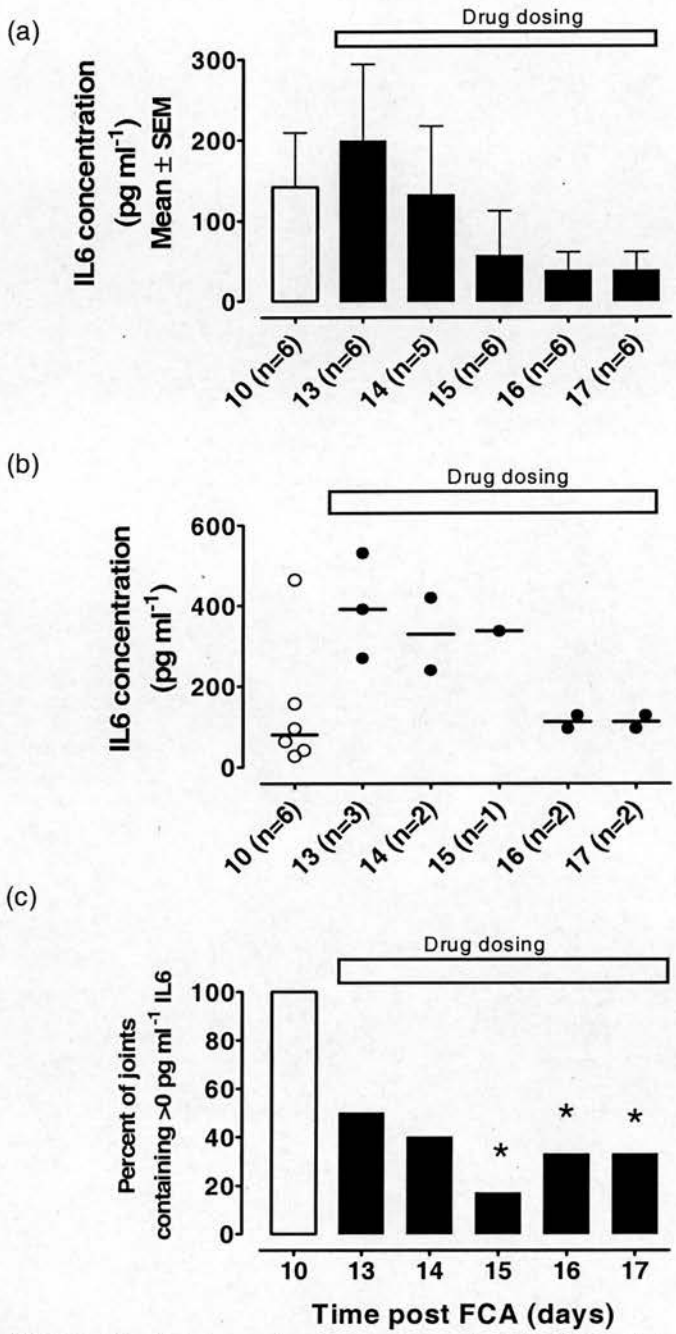


Figure 5.19 Levels of IL6 in (a) all joint perfusate samples and (b) in joints that were positive for IL6 content in pre-prednisolone treated rats (open symbols) and prednisolone-treated rats (closed symbols); the horizontal bar on the graph indicates the median concentration. (c) The percentage of FCA-injected (ipsilateral) knee joints that were positive for IL6, *i.e.* contained detectable levels of this cytokine. Statistical significance ($P<0.05$) is represented by *. Statistical analysis for (a) and (b) were carried out to compare concentrations on each day post FCA with levels on day 10 using the Kruskal Wallis test. The Fisher's Exact test was used to analyse the differences in proportion of samples that had detectable levels of the protein in (c).

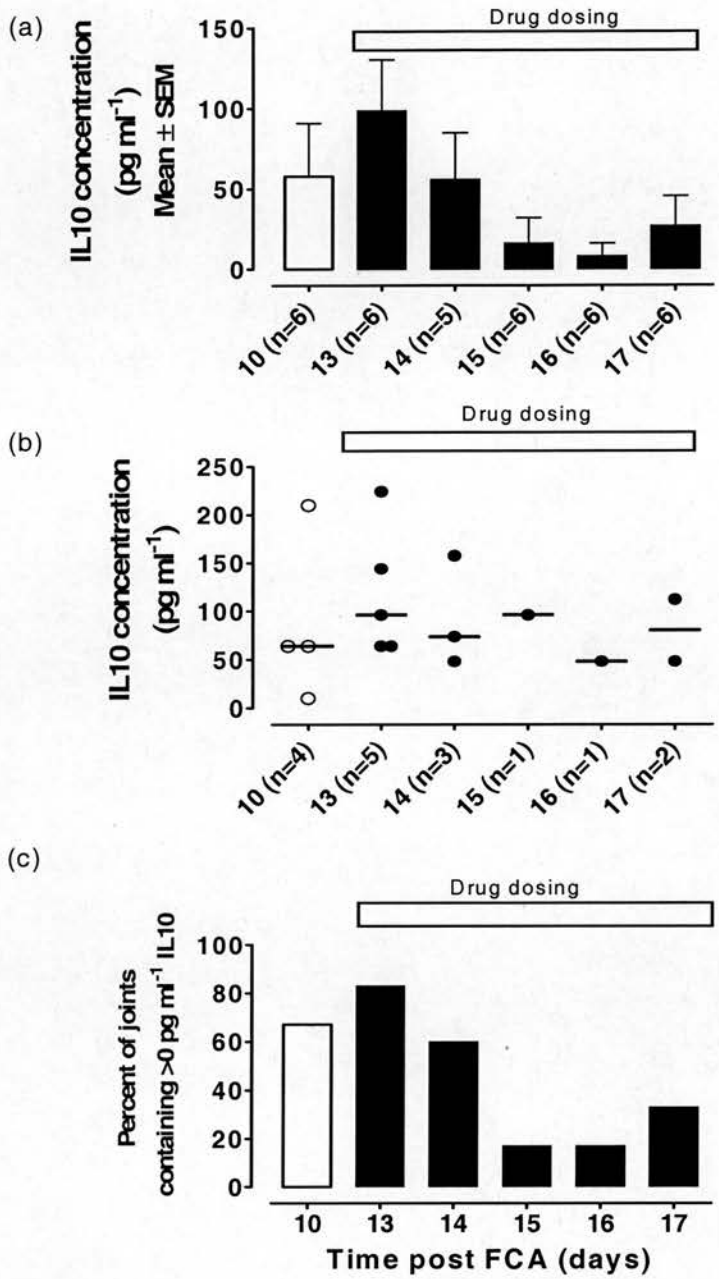


Figure 5.20 Levels of IL10 in (a) all joint perfusate samples and (b) in joints that were positive for IL10 content in pre-prednisolone treated rats (open symbols) and prednisolone-treated rats (closed symbols); the horizontal bar on the graph indicates the median concentration. (c) The percentage of FCA-injected (ipsilateral) knee joints that were positive for IL10, *i.e.* contained detectable levels of this cytokine. Statistical significance ($P<0.05$) is represented by *. Statistical analysis for (a) and (b) were carried out to compare concentrations on each day post FCA with levels on day 10 using the Kruskal Wallis test. The Fisher's Exact test was used to analyse the differences in proportion of samples that had detectable levels of the protein in (c).

Tumour Necrosis Factor α

No significant differences were observed between TNFα concentrations in ipsilateral (FCA-injected) joints on day 10 (31.8 ± 8.8 pg ml^{-1}; n=6; pre-prednisolone treatment) and any day during the drug-treatment regime (P>0.05, Kruskal Wallis; see Figure 5.21a). The TNFα content of TNFα-positive FCA-injected joints was not significantly different during prednisolone administration in comparison with the day 10, pre-treatment value (P>0.05, Kruskal Wallis). However, prednisolone administration significantly reduced the proportion of TNFα-positive joints on day 15 post-FCA (P>0.05, Fisher's Exact test; see Figure 5.21c).

Interleukin 2 and 4, Granulocyte–Macrophage CSF and Interferon γ

Concentrations of IL2, IL4, GM-CSF and IFNγ in ipsilateral joints of FCA-injected animals on day 10 (pre-prednisolone treatment) and during drug administration (days 13-17) are shown in Table 5.3. No significant differences between pre-treatment levels and values during prednisolone-treatment occurred (P>0.05, Kruskal Wallis).

Cytokine content of joints- Contralateral

In rats with established arthritis (day 10) perfusates from contralateral joints contained detectable levels of cytokines (see Figure 5.22). Treatment with prednisolone (10 mg kg^{-1}) had no significant effect (P <0.05, Kruskal Wallis).

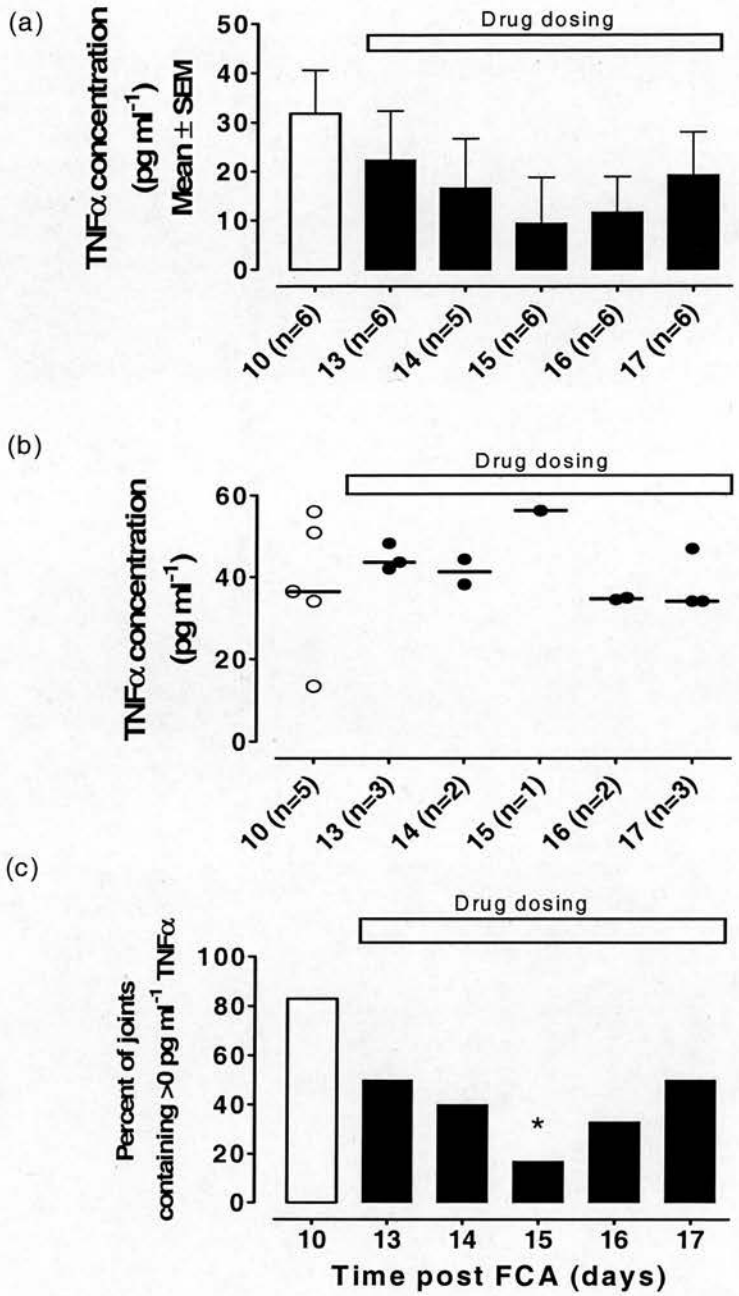


Figure 5.21 Levels of TNFα in (a) all joint perfusate samples and (b) in joints that were positive forTNFα content in pre-prednisolone treated rats (open symbols) and prednisolone-treated rats (closed symbols); the horizontal bar on the graph indicates the median concentration. (c) The percentage of FCA-injected (ipsilateral) knee joints that were positive for TNFα, *i.e.* contained detectable levels of this cytokine. Statistical significance ($P<0.05$) is represented by *. Statistical analysis for (a) and (b) were carried out to compare concentrations on each day post FCA with levels on day 10 using the Kruskal Wallis test. The Fisher's Exact test was used to analyse the differences in proportion of samples that had detectable levels of the protein in (c).

	Day	IL2	IL4	GM-CSF	IFNγ
Levels in ipsilateral joint perfusates (Mean ±SEM; pg ml^{-1})	**10**	3.3 ± 1.9 (n=6)	21.4 ± 9.9 (n=6)	10.5 ± 4.2 (n=6)	4.9 ± 2.3 (n=6)
	13	2.7 ± 0.9 (n=6)	41.6 ± 20.3 (n=6)	12.2 ± 5.5 (n=6)	8.8 ± 2.0 (n=6)
All data	**14**	3.6 ± 1.7 (n=5)	22.7 ± 15.4 (n=5)	7.8 ± 4.8 (n=5)	5.6 ± 2.3 (n=5)
	15	0.9 ± 0.9 (n=6)	11.6 ± 11.6 (n=6)	4.7 ± 4.7 (n=6)	2.1 ± 2.1 (n=6)
	16	0.5 ± 0.5 (n=6)	4.4 ± 4.4 (n=6)	5.6 ± 3.5 (n=6)	5.6 ± 1.8 (n=6)
	17	0.9 ± 0.9 (n=6)	3.8 ± 3.8 (n=6)	2.5 ± 2.5 (n=6)	6.2 ± 2.0 (n=6)
Levels in ipsilateral joint perfusates (Mean ±SEM; pg ml^{-1})	**10**	6.7 ± 2.7 (n=3)	42.8 ± 5.8 (n=3)	15.8 ± 3.9 (n=2)	9.9 ± 1.2 (n=3)
	13	4.1 ± 0.3 (n=4)	83.3 ± 17.9 (n=3)	24.4 ± 2.1 (n=3)	10.5 ± 1.2 (n=5)
Positive joints only	**14**	6.0 ± 1.7 (n=3)	56.8 ± 20.8 (n=2)	19.5 ± 1.3 (n=2)	9.4 ± 0.5 (n=3)
	15	5.3 ± 0.0 (n=1)	69.8 ± 0.0 (n=1)	28.2 ± 0.0 (n=1)	12.4 ± 0.0 (n=1)
	16	3.0 ± 0.0 (n=1)	26.3 ± 0.0 (n=1)	16.8 ±0.1 (n=2)	8.4 ± 0.1 (n=4)
	17	5.6 ± 0.0 (n=1)	22.5 ± 0.0 (n=1)	14.8 ± 0.0 (n=1)	9.4 ± 0.7 (n=4)
Percentage of positive joints	**10**	50	50	67	50
	13	66	50	50	83
	14	60	40	40	60
	15	17	17	17	17
	16	17	17	33	67
	17	17	17	17	67

Table 5.3 Concentrations of IL2, IL4, GM-CSF and IFNγ in FCA-injected (ipsilateral) knee joints before prednisolone treatment (grey bars) and during prednisolone administration (days 13-17). The values for levels in positive joints only and the percentage of joints that were positive for each of the cytokines are also shown. No significant changes occurred.

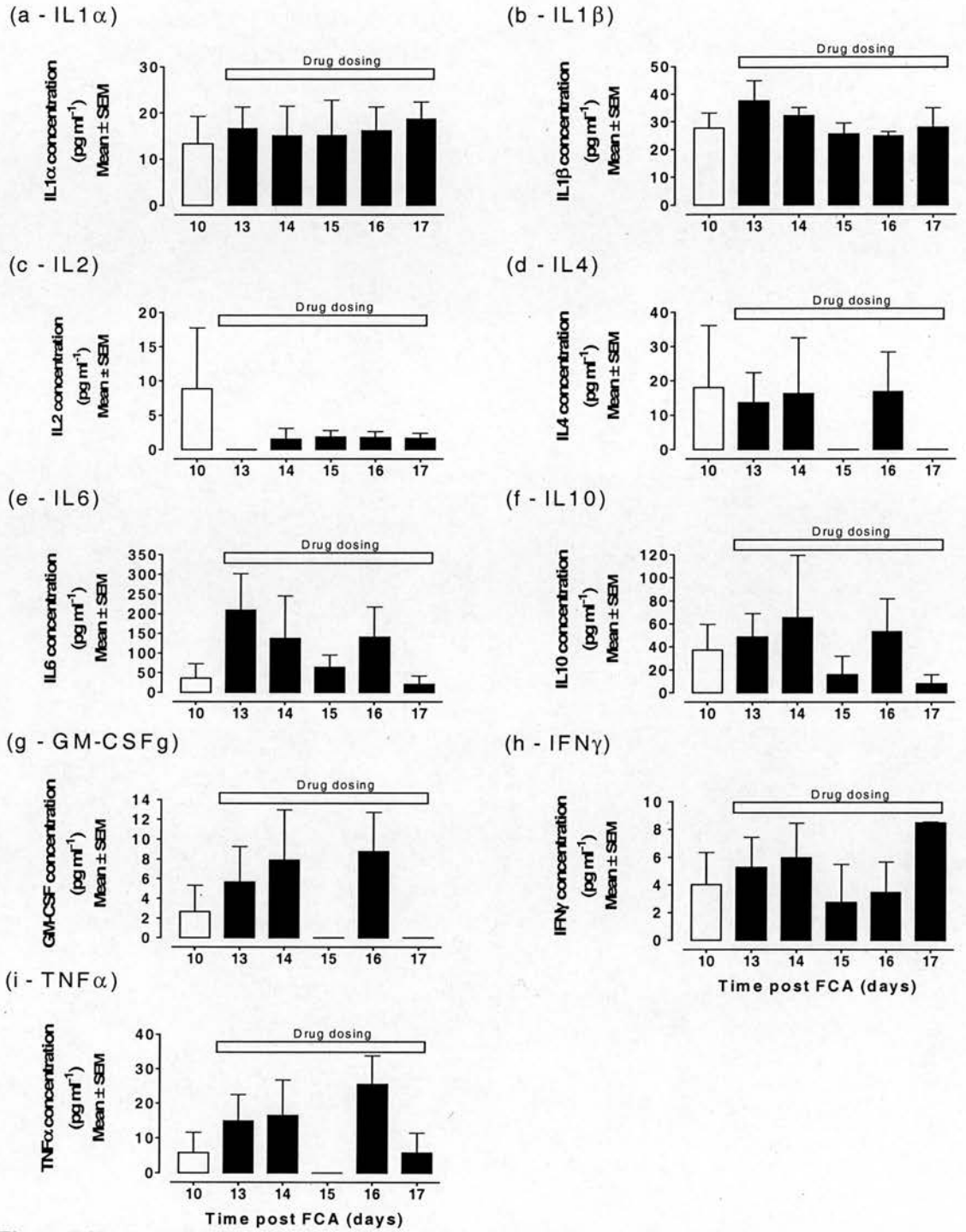


Figure 5.22 Levels of (a) IL1α, (b) IL1β, (c) IL2, (d) IL4, (e) IL6, (f) IL10, (g) GM-CSF, (h) IFNγ and (i) TNFα in contralateral (un-injected) knee joints from FCA-treated animals (n=6) before prednisolone treatment (day 10; open bars) and during prednisolone administration (days 13-17; closed bars). Statistical analysis was carried out to compare concentrations on each day during drug treatment with levels pre-treatment (day 10) using the Kruskal Wallis test.

5.4 DISCUSSION

The primary aim of this study was to identify temporal expression patterns of a) cytokines and b) inflammatory cells in the knee joints of rats following induction of arthritis using the novel perfusion technique, described in Chapter 4. Once it had been established that FCA significantly increased expression of the IL1α, IL1β, TNFα and GM-CSF and tended to increase (although not significantly) levels of IL6, IL10, IL2, IL4 and IFNγ, a second study was conducted during which a glucocorticosteroid, prednisolone, was administered daily. Joint perfusions were performed one hour later, in order to determine whether the anti-inflammatory steroid had an effect on the levels of inflammatory proteins induced by FCA. This time point was chosen for the perfusions as the maximum analgesia in the behavioural assessments was observed. The inclusion of vehicle-treated animals was considered, but was decided to be unnecessary in terms of controlling against the FCA-induced inflammation, due to the fact that the inflammatory process arising from the injection of FCA arises in part as a result of the injection itself, the increase in intra-articular pressure, the vehicle and the bacterial substance within the FCA.

Cytokine expression altered over time as FCA-induced arthritis progressed from the acute to the more "chronic" phase. The proportion of inflamed joints that contained detectable levels of all mediators (except IL4) measured was significantly increased during the study. This suggests that it may be the presence of the protein, even at low levels, that is important for the development and maintenance of joint inflammation and hypersensitivity. In addition, significant correlations between measures of joint swelling or mechanical hypersensitivity and levels of cytokines in inflamed joints were seen. Furthermore, trends towards increased concentrations of all mediators measured in blood plasma were elevated in comparison to normal joints, although none of these were found to correlate with behavioural signs of arthritis.

Cytokines are locally-produced proteins known to be involved in many biological functions, including inflammation, bone remodelling, cell growth and activation (for review see Christodoulou & Choy, 2006). It is therefore not surprising that they play a role in joint inflammation during autoimmune diseases such as RA, and that anti-cytokine therapies are being successfully used in the clinic to slow the progression of the disease and joint destruction and provide effective analgesia.

INTERLEUKIN 1α

IL1α levels remained significantly elevated for the duration of the experiment, indicating that IL1α may be involved in sustaining the inflammatory response in this model following the initial insult. Furthermore, between 80 and 100% of joints contained detectable levels of this protein at all time points post-FCA, this implies that IL1α is important for the development of adjuvant-arthritis. In accord, the transgenic mouse line which overexpresses human IL1α exhibits a severe arthritic phenotype (Niki *et al.*, 2004). Furthermore, levels of IL1α in the inflamed joint were strongly correlated with functional measures of mechanical hypersensitivity and joint swelling. Similarly, in patients with RA, plasma IL1α levels significantly correlate with the Ritchie Articular Index, analogue pain scores and duration of morning stiffness (Eastgate *et al.*, 1991). However, IL1β mRNA and protein production greatly exceeds that of IL1α clinically in RA joints (Dinarello, 1994c). The levels of IL1α protein in joint exudates from arthritic rats in this study are in agreement; the maximum levels of the two cytokines were 60 pg ml^{-1} and 400 pg ml^{-1} respectively.

INTERLEUKIN 1β

In contrast to IL1α, significantly increased IL1β expression occurred only in the acute phase and declined toward basal values within ten days, despite the persistence of the clinical symptoms. However, the proportion of joints that were IL1β-positive was high throughout the study, between 60 and 100% and hence it is a valid drug target effective in a high proportion of

patients, perhaps those that do not respond to TNFα therapy. A strong correlation between levels of IL1β in the joint and the swelling occurred, moreover, IL1β antagonists reduce swelling of arthritic joints in patients (Bresnihan *et al.*, 1998). The level of IL1β in inflamed joints was strongly correlated with mechanical hypersensitivity, which is in agreement with similar studies in patients (Alstergren *et al.*, 1998). In patients with RA, levels of IL1β in synovial fluid was approximately 360 pg ml^{-1} (McNiff *et al.*, 1995), which are comparable to levels measured in the joint perfusates of adjuvant-injected rat joints during the first week of joint inflammation.

INTERLEUKIN 6

IL6 protein and mRNA have been detected in human RA synovial tissue (Hirano *et al.*, 1988; Houssiau *et al.*, 1988), although no statistically significant increases in IL6 protein were measured at any time point in this study, there was a trend showing that the peak protein expression was in the acute phase and levels remained very low thereafter. Moreover, no correlation between levels in the joint or in plasma and joint swelling was observed, although IL6 plasma levels have been previously shown to correlate with footpad swelling in adjuvant-induced arthritic Lewis rats treated with cyclosporin A or indomethacin (Leisten *et al.*, 1990). However, IL6 in perfusates was highly correlated with mechanical hypersensitivity in the rats suggesting it may be directly involved in the development of alterations in "nociception" as a result of joint inflammation (for a review on the role of IL6 in pain and nociception see De Jongh *et al.*, 2003). Although IL6 is known to be a key mediator in joint inflammation and RA, less than 50% of inflamed joints contained the protein at any time point during this study. In a double-blind randomised controlled clinical trial treating RA patients with the IL6 receptor antagonist, tocilizumab, a maximum of 63% of patients achieved American college of rheumatology 20 (ACR; see below; McQuay & Moore, 2002) with tocilizumab monotherapy (Maini *et al.*, 2006). This could suggest that IL6 may not have had a predominant role in the rest of the RA population of patients.

The ACR endpoint for effective treatment of RA states that patients must have:

1. 20% improvement in tender joint count
2. 20% improvement in swollen joint count
3. 20% improvement in three of the following:
    - Global disease activity assessed by observer
    - Global disease activity assessed by patient
    - Patient assessment of pain
    - Physical disability score (like health assessment questionnaire)

Levels of IL6 in inflamed joints in the present study are comparable to that seen clinically in RA patients; the levels have been reported to be between 160 and 600 pg ml^{-1} in human synovial fluid (Matsumoto *et al.,* 2006; McNiff *et al.,* 2006), whereas the peak expression of IL6 protein was approximately 200 pg ml^{-1} in rat joints following FCA. Therefore, although IL6 reduction by novel therapies may improve RA, it is likely there will be a proportion of "non-responders", as there is for current TNFα and IL1β antagonists. Perhaps a combination of several cytokine antagonists would prove to be more efficacious and have more widespread benefit to more patients, although this may result in increased risk of infection due to more widespread immunosuppression.

TUMOUR NECROSIS FACTOR α

TNFα, like IL1β, is present in synovial fluid of patients with RA (Di Giovine *et al.*, 1988; Hopkins & Meager, 1988). Similar levels of TNFα protein in collagen-injected mouse joints were seen as in this study; the levels in the mouse joints peaked at 35 pg ml^{-1} (Rioja *et al.*, 2004). The concentrations detected in the present study were lower, but Rioja *et al* used whole homogenised joints, including surrounding tissue as well as synovial fluid. It would therefore be expected that values should be higher, taking into account all TNFα in the joint and synovial cavity. Moreover, clinically levels of TNFα in synovial fluid from patients with RA have been

reported between 13 and 562 pg ml^{-1} (McNiff *et al.*, 1995; de Jager *et al.*, 2006), whereas levels in the present study, measured in joint perfusate samples peaked at approximately 15 pg ml^{-1}. Levels of TNFα in inflamed joints in the present study were significantly correlated with joint swelling and hypersensitivity. In accord, neutralising monoclonal antibodies of TNFα reduce joint swelling in rabbits with antigen induced arthritis (Lewthwaite *et al.*, 1995) and inhibition of TNFα production abolished ankle joint swelling in rats with established experimental arthritis (Conway *et al.*, 2001). Other studies have also shown the importance of TNFα in the development of inflammatory hyperalgesia and carageenan-induced knee joint pain (Cunha *et al.*, 1992; Junger & Sorkin, 2000; Tonussi & Ferreira, 1999). The proportion of joints that were TNFα-positive was dramatically increased during the first two weeks of the study, between 60 and 80%. TNFα antagonists provide impressive protection against pain and joint swelling in most RA patients (Maini & Taylor, 2000; Charles *et al.*, 1999; Edwards, 1999; Fox, 2000; Garrison & McDonnell, 1999; Kavanaugh *et al.*, 2000; Lorenz *et al.*, 2000; Moreland *et al.*, 2000), however, it is also evident that anti-TNFα therapy is not effective in all RA patients (Smolen & Steiner, 2003), nor does it control arthritis in all affected joints of good responders.

The lower levels of expression of the three key pro-inflammatory cytokines, IL1β, TNFα and IL6, in the "chronic" phase during this study suggest other factors may maintain this phase of the response. Other studies have followed the progression of FCA-induced joint inflammation for up to 90 days, and the physical symptoms persist throughout this duration (Wilson *et al.*, 2006). However, it may be that higher levels of specific mediators are needed to induce joint inflammation or arthritis but that lower levels are sufficient to maintain the inflammation.

ANTI-INFLAMMATORY CYTOKINES

IL4 and IL10, Th2 helper cell-derived cytokines, have been previously demonstrated in RA joints, although the expression levels of IL4 are relatively low, despite the abundance of T

cells (Chen *et al.*, 1993; Miyata *et al.*, 2000; Simon *et al.*, 1994; Ulfgren *et al.*, 1995). In other animal models of arthritis, IL4 levels have been found to be low or negligible, in agreement with our results (Schmidt-Weber *et al.*, 1999). It is possible that the lack of IL4 –producing CD4+ Th2 cells contributes to the pathogenesis of RA, and this has led to suggestions that IL4 may be a useful therapeutic agent (Miossec *et al.*, 1990).

In contrast IL10 had increased levels (although not statistically significant) of expression throughout the disease course in this study, which is in agreement with human studies of RA-affected joints (Wagner *et al.*, 1997). IL4 and IL10 have been employed in clinical trials for the treatment of RA and despite efficacy in animal models (Tarner *et al.*, 2002), a Phase I trial of recombinant IL4 in RA, although well tolerated, did not reveal significant clinical benefit (van den Bosch, 1998). In contrast, IL10 initially seemed to be beneficial (Weinblatt, 1999) and led to pronounced increases of circulating TNFα receptors and IL1Ra (Maini *et al.*, 1997). However, thrombocytopenia occurred in some patients and efficacy was not discerned in subsequent studies. The development of both IL4 and IL10 for RA was discontinued but it may still be possible that the combination of both cytokines, or other T helper cell-derived products, could lead to a more marked improvement (van Roon *et al.*, 2002). Alternatively, local administration, for example into the joint space, may reduce the associated side effects, whilst still providing efficacious improvement of RA.

CONTRALATERAL SPREAD OF CYTOKINE EXPRESSION

Surprisingly, contralateral joints also showed increased expression of the nine cytokines measured, although absolute levels remained lower than in the inflamed joint at all time points. Contralateral joint inflammation as a result of a unilateral inflammatory insult is a well documented occurrence (Donaldson, 1999; Shenker *et al.*, 2003), although behavioural signs of this crossover are not usually evident with the dose of adjuvant used here (Donaldson *et al.*,

1993). It may be that the inflammation which occurs in the contralateral knee joint is not sufficient to initiate swelling or hypersensitivity. Histological processing from this joint has also shown that no changes are seen in the contralateral joint in rats injected with 150 μg FCA (previous unpublished work from this laboratory). However, pain and inflammation of the joint arises not only from cytokines within the joint, but via neural mechanisms peripherally and centrally, which may explain the lack of symptoms in the contralateral limb, despite the fact that inflammatory proteins and cells are present within the joint. Alternatively, mild discomfort in the contralateral joint may exist but is outweighed by more severe hypersensitivity in the inflamed joint. When higher doses of FCA are used, neural mechanisms may result in more widespread hypersensitivity and oedema. The "on" and "off" cells in the rostral ventromedial medulla (RVM) of the brain are involved in pain processes. "Off" cells exert net inhibitory effect on nociception, they cease firing prior to withdrawal to noxious heat. In contrast "on" cells have a net facilitatory effect on nociception; they begin firing immediately prior to limb withdrawal to a noxious heat stimulus. The organisation of the RVM suggests that "on" and "off" cells function as a unit that exerts global, rather than topographical, discrete control over pain transmission. This may help explain mild sensitisation and contralateral effects, which may exist in other joints of the body, but were only noted here only in the knee joint, as no other areas were studied.

EFFECT OF PREDNISOLONE ON JOINT INFLAMMATION

Prednisolone did not affect the absolute levels of cytokines in inflamed joints, although it reduced the percentage of inflamed joints that contained detectable levels of IL1α and IL6. This suggests that the steroid appears to have an all-or-none effect in terms of cytokines. This is in contrast to other studies, in which prednisolone reduced joint swelling through a mechanism associated with a reduction in IL1β and IL6 protein and mRNA expression levels in CIA and

SCW arthritis (Rioja *et al.*, 2004). However, Rioja and colleagues administered prednisolone immediately after induction of joint arthritis, therefore perhaps preventing the production of cytokines, rather than stopping or reducing their expression once already present when administered over a five day period. Clinically, effective prednisolone treatment is associated with a marked reduction in macrophage infiltration in the RA synovial tissue after two weeks of treatment (Gerlag *et al.*, 2004; Haringman *et al.*, 2005). Although no inflammatory cell counts were measured here, their inflammatory products were, and were not reduced. The predominant effect of corticosteroids is to switch off multiple inflammatory genes (encoding cytokines, chemokines, adhesion molecules, inflammatory enzymes, receptors and proteins) that have been activated during the chronic inflammatory process. At higher concentrations glucocorticoids have additional effects on the synthesis of anti-inflammatory proteins and postgenomic effects (Barnes, 2006). Perhaps the duration of prednisolone administration was not sufficient in the present study to see effects on inflammatory mediators, despite the fact that the swelling of the joint was reduced within the week. This could be confirmed by dosing rats with established arthritis with prednisolone over a longer period, several weeks, perfusing the joints and assaying the perfusate for cytokines. Additionally, the n numbers used here were relatively low (six per day), if these were increased, more effect may be seen on absolute cytokine levels, however due to ethical reasons, the aim of reducing animal use, as well as financial and time limitations, this was not possible for the purpose of this thesis.

SUMMARY

In conclusion, these studies have demonstrated the application of the novel joint perfusion method for sampling synovial fluid and assayed the perfusates for cytokines over a three week period of adjuvant-induced arthritis. As well as revealing additional information regarding the expression patterns of cytokines during adjuvant-induced arthritis, similarities

between this animal model of joint disease and human RA have been demonstrated that further validate the model as a valuable pre-clinical tool to study the inflammatory process of human RA. Moreover, consolidation of these similarities helps improve the confidence of novel drug screening using this model prior to use in the clinic.

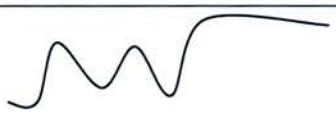
Cytokine	Time course of expression in synovial joint space (over 21 days)	Significant correlation to behavioural measure of...	
		Swelling	Mechanical hypersensitivity
IL1α		✓	✓
IL1β		✓	✓
IL6		X	✓
IL10		✓	X
TNFα		X	✓
IL2		X	X
IL4		✓	X
GM-CSF		X	X
IFNγ		X	X

Table 5.4 A summary of changes in cytokine expression profiles within adjuvant-injected rat knee joints and the important relationships between these levels and behavioural measures of joint swelling and hypersensitivity

Chapter 6 – The role of IL1β in joint pain and inflammation

6.1 INTRODUCTION

IL1β is a key mediator of synovial inflammation, responsible for

- Triggering the recruitment of PMNs, lymphocytes and monocytes into the joint
- Activation of macrophages, resulting in release of proteases (Bresnihan *et al.*, 1998)
- Stimulation of T- and B-cell proliferation and differentiation (Dinarello, 2002)
- Enhancing the proliferation of fibroblasts, leading to pannus formation.

IL1β plays an important role in the pathophysiology of joint inflammation and RA by contributing to the inflammatory process as well as joint destruction; levels of IL1β are higher in joint tissue biopsies from patients with joint erosions than those with non-erosive RA (Fong *et al.*, 1994). Inhibition of IL1β with recombinant IL1Ra reduces the extent of inflammation in RA in patients (Bresnihan *et al.*, 1998) and lessens inflammation and bone resorption in various experimental models of arthritis (Feige *et al.*, 2000; Joosten *et al.*, 1996; Kuiper *et al.*, 1998; Wooley *et al.*, 1993a; Wooley *et al.*, 1993b).

Infusion of IL1β (s.c.) induces and exacerbates arthritis in mouse and rat models of joint inflammation (Hom *et al.*, 1988; Hom *et al.*, 1990; Schwab *et al.*, 1991). Furthermore, i.art injection of IL1β in rabbits induces a transient infiltration of neutrophils into the joint space, followed by mononuclear cell infiltration which lasts a few days, and loss of proteoglycans from the articular cartilage (Pettipher *et al.*, 1986). In addition, i.art IL1β induced a significant dose-dependent increase in inflammation, bone resorption and the number of osteoclasts in rat knee joints and was accompanied with mild cartilage degradation (Bolon *et al.*, 2004).

In contrast, it has also been reported that injection of IL1β into the knee joints of rats with established adjuvant-induced arthritis reduced inflammation and joint destruction (Jacobs *et al.*, 1988). This effect was observed with administration of IL1β prior to adjuvant and administration during established arthritis. Furthermore, it has been shown that i.v. or s.c. IL1β inhibits carageenan-induced paw oedema in a dose-dependent manner in rats (Drelon *et al.*,

1994; Drelon *et al.*, 1992; Nakamura *et al.*, 1988). The inhibition of oedema is concomitant with the increase of plasma levels of adrenocorticotophic hormone and corticosterone, hence anti-inflammatory actions of IL1β may be, at least in part, explained by pituitary-adrenal axis simulation (Nakamura *et al.*, 1988). It has also been suggested that the anti-inflammatory effect of pro-inflammatory cytokines injected into inflamed tissues is mediated by the liberation of endorphins from inflammatory cells (Czlonkowski *et al.*, 1993).

The aims of this study were to:

- Determine whether IL1β could induce acute and/or "chronic" joint swelling, hypersensitivity and joint destruction
- To assess the release of other pro- and anti-inflammatory cytokines as a result of i.art IL1β
- To determine the effect of IL1β on primary sensory afferent neural activity and sensitivity using *in vivo* electrophysiological recording.

6.2 METHODS

All methods were carried out as described in Chapter 2.

Perfusion of the knee joint in anaesthetised rats was carried out as described in Section 2.4. To determine the acute effects of IL1β on the release of pro- and anti-inflammatory cytokine release, the left knee joints of six normal (untreated) rats were perfused to collect basal samples. Then recombinant rat IL1β (1 μg in 100 μl; n=6) or vehicle (100 μl sterile saline; n=6) was perfused into the joint over one minute. One, two, three, four, five, six and seven hours later a 250 μl sample was collected via perfusion of the joint, the sample was frozen and stored at -20° C.

To evaluate the effects of IL1β on the cytokine content of the joint over a ten day period, IL1β (1 μg in 100 μl; n=15) or vehicle (100 μl sterile saline; n=15) were administered via i.art injection under transient halothane anaesthesia (3% in oxygen). Animals were allowed to recover, and the ipsilateral (injected) knee joints of three rats were perfused on days 1, 2, 4, 7 and 10 post-IL1β (n=3 per day) or vehicle (n=3 per day). Total inflammatory cell counts of joint perfusate samples were carried out as described in Section 2.4.2, samples were then frozen and stored at -20° C and later assayed for a range of nine cytokines (IL1α, IL1β, IL2, IL4, IL6, IL10, GM-CSF, IFNγ and TNFα) using the Luminex assay as described in Section 2.4.3.

6.3. RESULTS

6.3.1. EFFECT OF IL1β ON BASAL NEURAL DISCHARGE OF PRIMARY AFFERENT NERVES

The basal neural discharge frequency of primary afferents innervating normal rat knee joints was 0.01 ± 0 impulses s^{-1} (n=13 rats, 32 units; 60% of units were inactive at this time). This increased to 0.08 ± 0.04 (n=8 rats; 23 units; 40% of units were inactive at this time) or 0.07 ± 0.04 impulses s^{-1} (n=5 rats; 9 units; 55% of units were inactive at this time) 30 minutes after i.art IL1β (0.1 μg) or vehicle respectively, these were not significantly different (P>0.05, Mann Whitney). Between 30 and 150 minutes the basal neural discharge frequency declined towards basal levels in vehicle-injected joints but increased towards a peak 180 minutes after IL1β administration. The frequency was 0.26 ± 0.06 impulses s^{-1} (n=8 rats, 23 units; 0% of units were inactive at this time), which was significantly higher than that of vehicle-treated joints at this time point (P<0.05, Mann Whitney; see Figure 6.1).

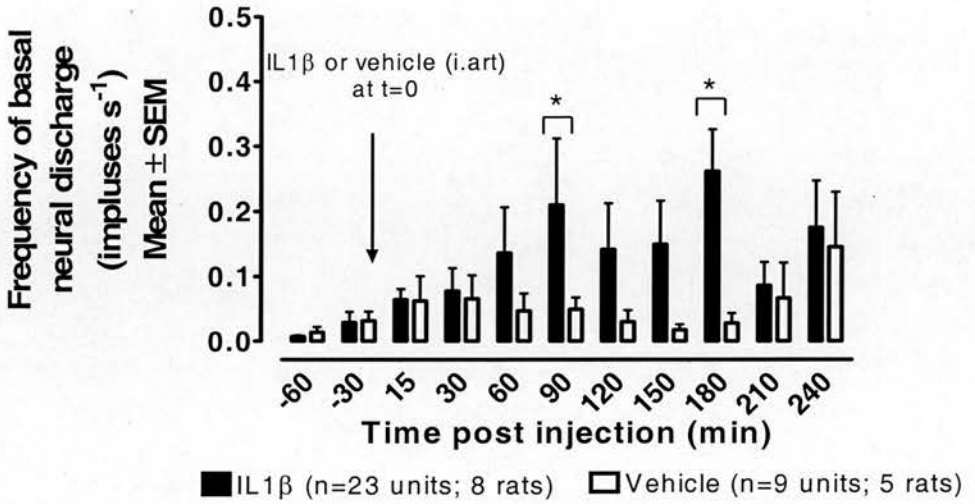


Figure 6.1 Basal neural activity (impulses s^{-1}) in fibres of the MAN in normal rat knee joints injected with IL1β (n=23 units; 8 rats; 0.1 μg; closed bar) or vehicle (n=9 units; 5 rats; open bar; sterile saline) at time t=0 (arrow). IL1β resulted in a significant increase in spontaneous action potential discharge frequency in comparison with vehicle-injected joints 180 minutes after administration (* $P<0.05$, Mann Whitney).

6.3.2 EFFECT OF IL1β ON THE MECHANICAL THRESHOLD OF PRIMARY AFFERENT NERVES

The mechanical threshold for activation of primary afferent nerves innervating the normal rat knee joint was 5.20 ± 0.64 g (n=13 rats, 32 units). Following IL1β there was a significant reduction in the threshold to mechanical stimulation with von Frey airs in comparison with vehicle-injected joints between 60 and 210 minutes post injection ($P<0.05$, two-way ANOVA). However, by 240 minutes the effect had diminished (see Figure 6.2). The afferents included in this analysis probably include a mixture of nociceptors and low threshold mechano-sensitive afferents.

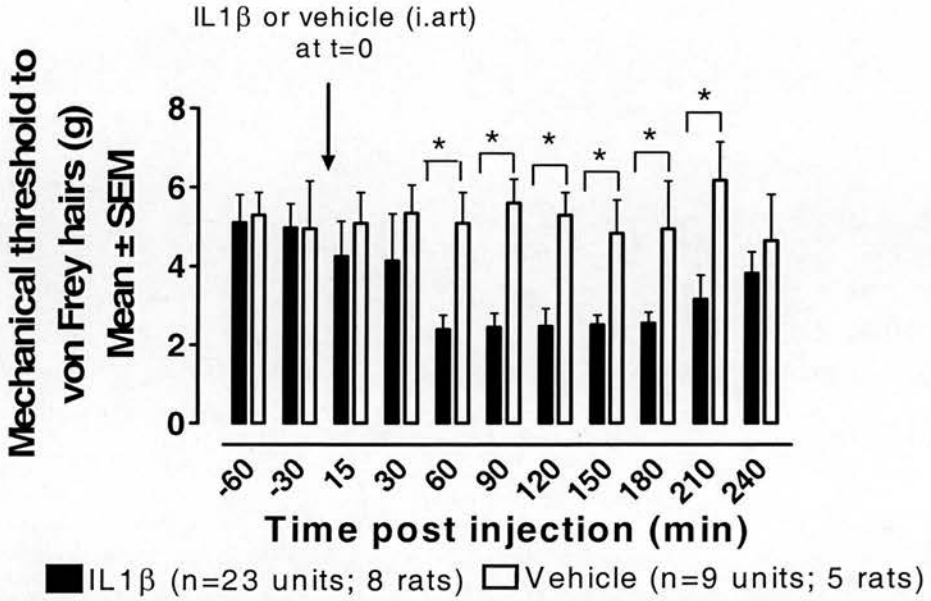


Figure 6.2 Mechanical threshold of primary afferent nerves in normal rat knee joints injected with IL1β (n=23 units; 8 rats; 0.1 μg; closed bar) or vehicle (n=9 units; 5 rats; open bar; sterile saline) at time t=0 (arrow). IL1β resulted in a significant decrease in mechanical threshold to von Frey hairs in comparison with vehicle-injected joints between 60 and 210 minutes after injection (* $P<0.05$, Two-way ANOVA).

6.3.3 EFFECT OF IL1β ON DISCHARGE FREQUENCY AND ACTION POTENTIAL COUNT TO MECHANICAL STIMULATION

The evoked response of primary afferents innervating the normal rat knee joints to 0.98, 12.5 and 20.9 g von Frey hairs were 0.05 ± 0.03, 2.69 ± 0.66 and 3.63 ± 0.95 impulses s^{-1} respectively (n=12 rats; 35 units). Following IL1β (0.1 μg; n=6) or vehicle (n=6), no significant changes to the evoked discharge frequency or total action potential count occurred ($P>0.05$, two-way ANOVA; see Figure 6.3).

6.3.4 EFFECT OF IL1β ON JOINT SWELLING AND MECHANICAL HYPERSENSITIVITY

I.art IL1β had no adverse effects on the animals' general health as demonstrated by the fact that the animals continued to feed and gain weight normally throughout the study (see

Figure 6.4a). Four, five, six and seven days post-IL1β the body weights of the rats were significantly higher than day 0 ($P<0.05$, Kruskal Wallis). There was no significant difference in body weight between the IL1β and vehicle-injected animals at any time point ($P>0.05$, two-way ANOVA).

Joint swelling

Before the i.art injection, the ipsilateral and contralateral knee joint diameters were 9.1 ± 0.1 mm (n=24) and 9.1 ± 0.1 mm (n=24) respectively, which were not significantly different ($P>0.05$, Mann Whitney). Following IL1β (1, 3 or 10 μg; n=6 at each dose) there was no significant difference in joint diameter in comparison with those of vehicle-injected joints (n=6) at any time point ($P>0.05$, two-way ANOVA; see Figure 6.4b). No changes in contralateral joint diameters occurred in comparison to the basal values at any time during this study ($P>0.05$, Kruskal Wallis).

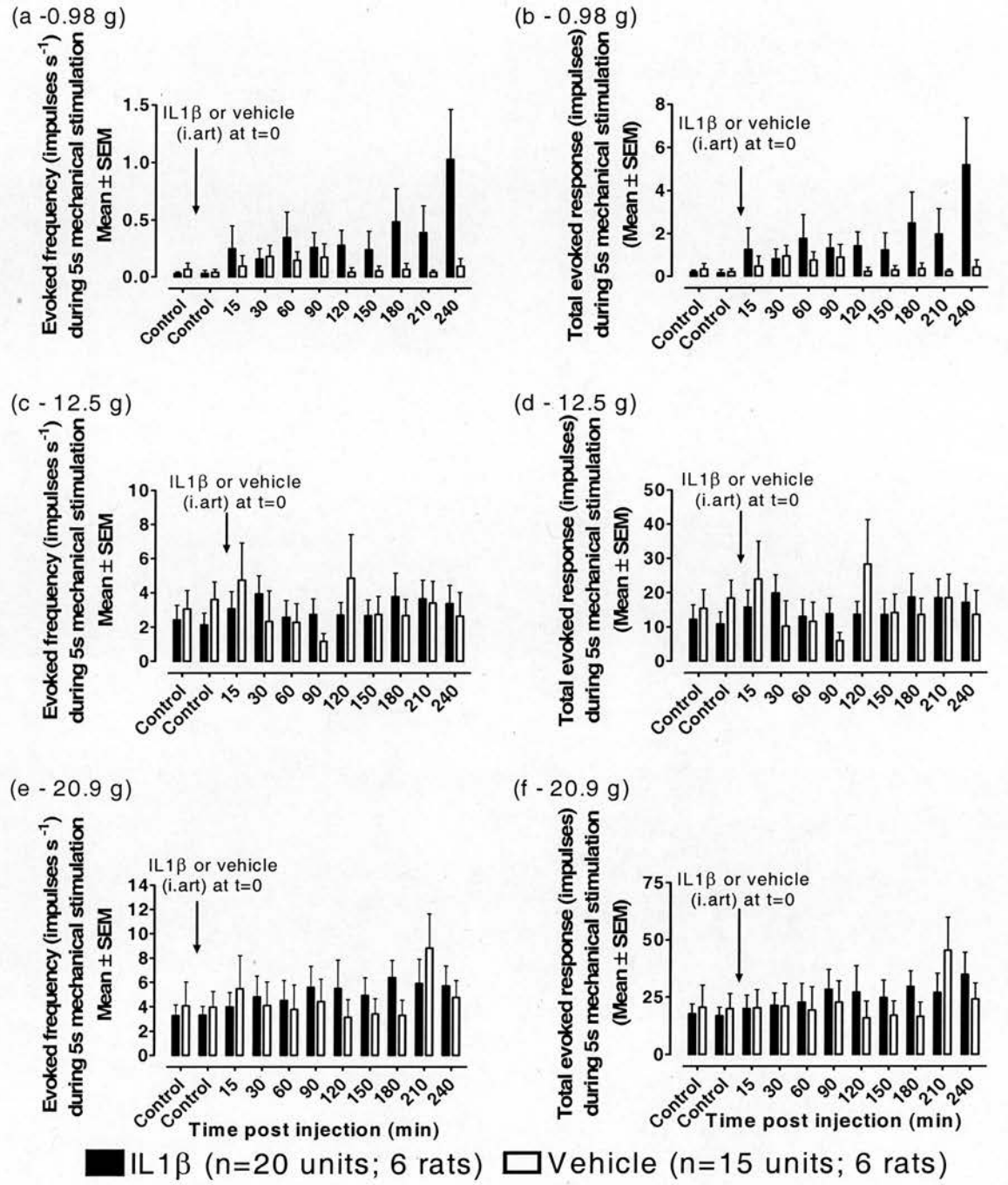


Figure 6.3 The evoked frequency and total action potential count during five seconds of mechanical stimulation of primary afferent nerves innervating normal rat knee joints. Stimulation with (a) and (b) 0.98 g, (c) and (d) 12.5 g and (e) and (f) 20.9 g von Frey hairs in normal joints and following IL1β (n=20 units; 6 rats; 0.1 μg; closed bar) or vehicle (n=15 units; 6 rats; open bar; sterile saline) at time t=0. IL1β had no effect on the evoked discharge frequency to mechanical stimulation by these von Frey hairs in comparison with vehicle-injected joints (P>0.05, two-way ANOVA).

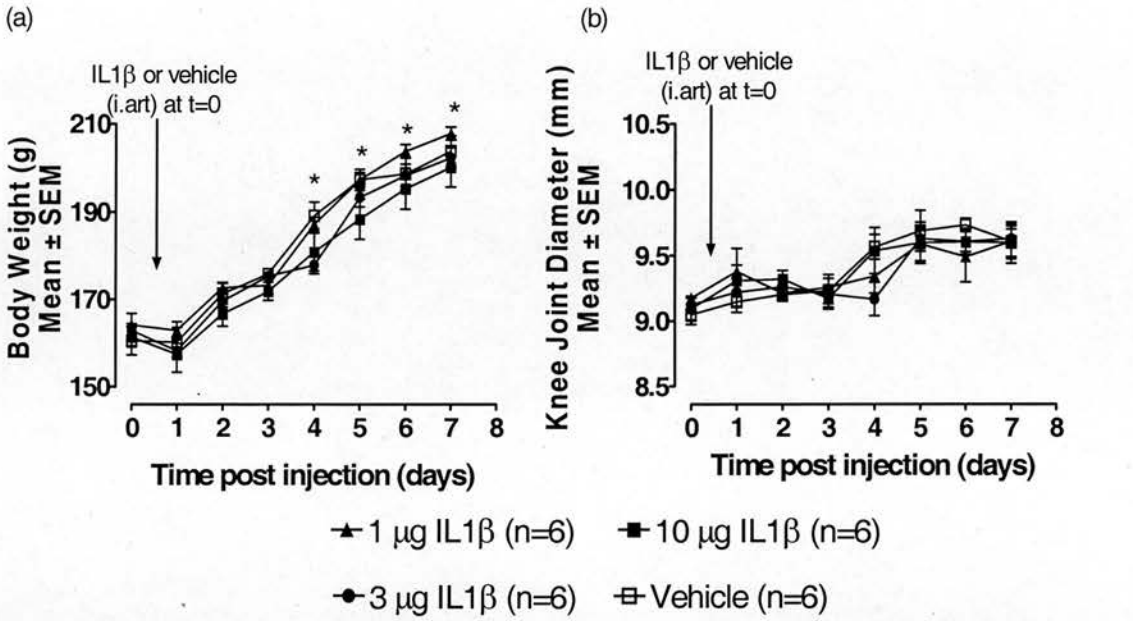


Figure 6.4. The (a) body weight and (b) ipsilateral knee joint diameters of IL1β- and vehicle-injected rats over a seven day study. IL1β (1, 3 or 10 μg; n=6) had no effect on ipsilateral or contralateral knee joint diameters in comparison with vehicle-injected (sterile saline) rats at any time during this study (n=6; * $P>0.05$, two-way ANOVA). Statistical analysis was performed to compare the body weight or joint diameter of the two groups on each day using the two-way ANOVA and a Bonferroni post-hoc test.

Mechanical hypersensitivity measured by the incapacitance tester

Prior to injection of IL1β, the weights on the two hind limbs were 65.0 ± 3.3 g (n=24) and 62.4± 2.8 g (n=24), with an associated ratio (ipsi:contra) of 1.1 ± 0.0. However, following administration of 1, 3 and 10 μg IL1β, the weights on the ipsilateral and contralateral hind limbs were significantly different from each other until 96 hours post-injection ($P<0.05$, Kruskal Wallis; see Figure 6.5 a, b and c). The associated ratio of the weight distribution between the hind limbs decreased by 23.6 ± 0.1 (n=6), 52.8 ± 0.1 (n=6) and 36.4 ± 0.1 % (n=6) in rats treated with 1, 3 and 10 μg IL1β respectively by four hours post administration. The ratio remained significantly reduced in comparison with vehicle-injected joints (n=6) until four days post-IL1β ($P<0.05$, two-way ANOVA; see Figure 6.5d).

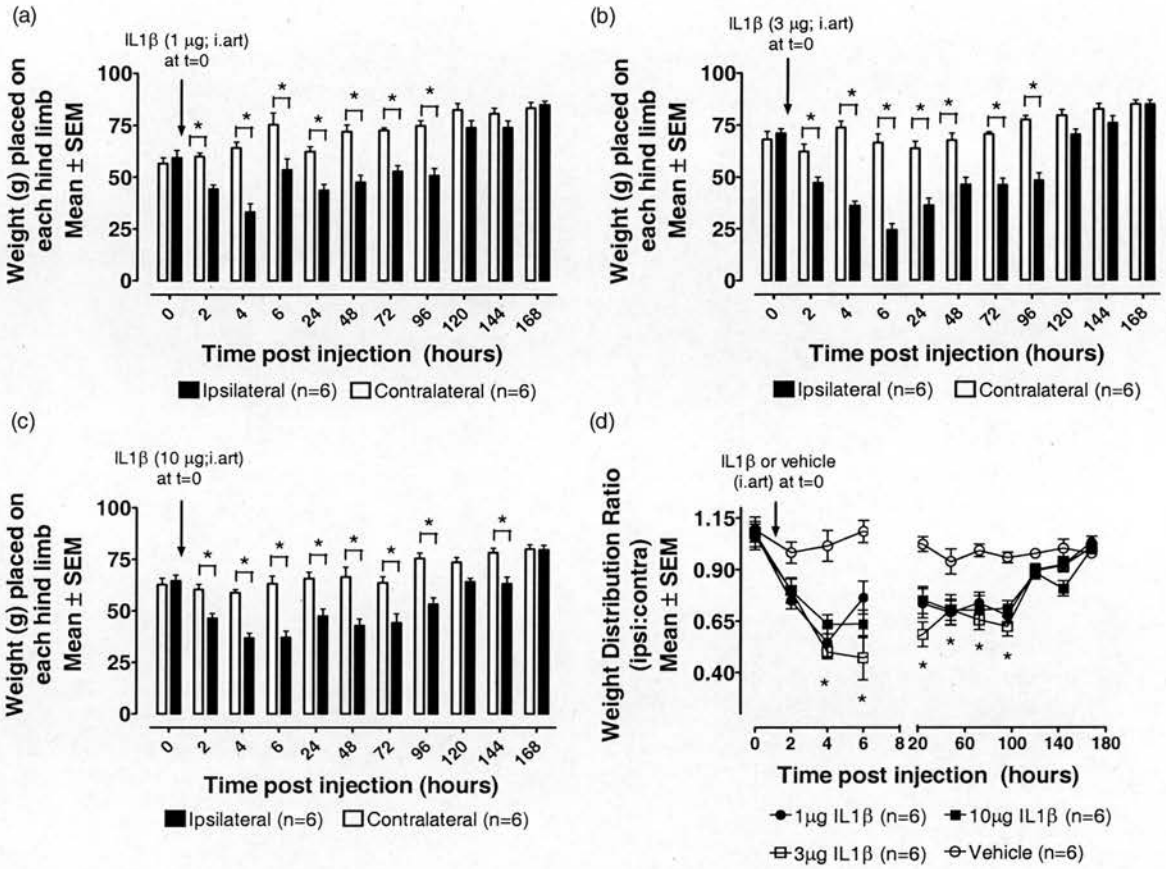


Figure 6.5 The absolute weight placed on the ipsilateral (injected) and contralateral hind limbs of rats injected with (a) 1 μg, (b) 3 μg and (c) 10 μg IL1β (n=6 at each dose) on day 0 after basal measurements were made and (d) the associated ratio of weight distribution between the hind limbs for IL1β- and vehicle-injected (n=6; sterile saline) rats. All doses of IL1β caused significant mechanical hypersensitivity of the joint from two hours post administration (* $P<0.05$, two-way ANOVA). Statistical analysis was performed to determine whether the difference between the weight on the ipsilateral and contralateral hind limbs were significantly different (a, b, and c) or whether the ratio of weight distribution was significantly reduced in IL1β-injected rats compared with vehicle-injected animals using a two-way ANOVA.

Mechanical hypersensitivity measured by PAD

Before induction of inflammation, the ipsilateral and contralateral LWTs were 409.4 ± 13.19 gf (n=24) and 443.0 ± 19.2 gf (n=24) respectively, which were not significantly different ($P>0.05$, Kruskal Wallis). However, following IL1β (1, 3 and 10 μg; n=6 at each dose) the ipsilateral LWTs were significantly reduced in comparison with vehicle-injected rats ($P<0.05$, two-way ANOVA; see Figure 6.6). The ipsilateral LWTs remained significantly reduced until

seven, five and 24 hours post IL1β for 1, 3 and 10 μg respectively ($P<0.05$, two-way ANOVA). No changes in the LWT of contralateral joints were observed at any time during this study ($P>0.05$, two-way ANOVA, see Table 6.1).

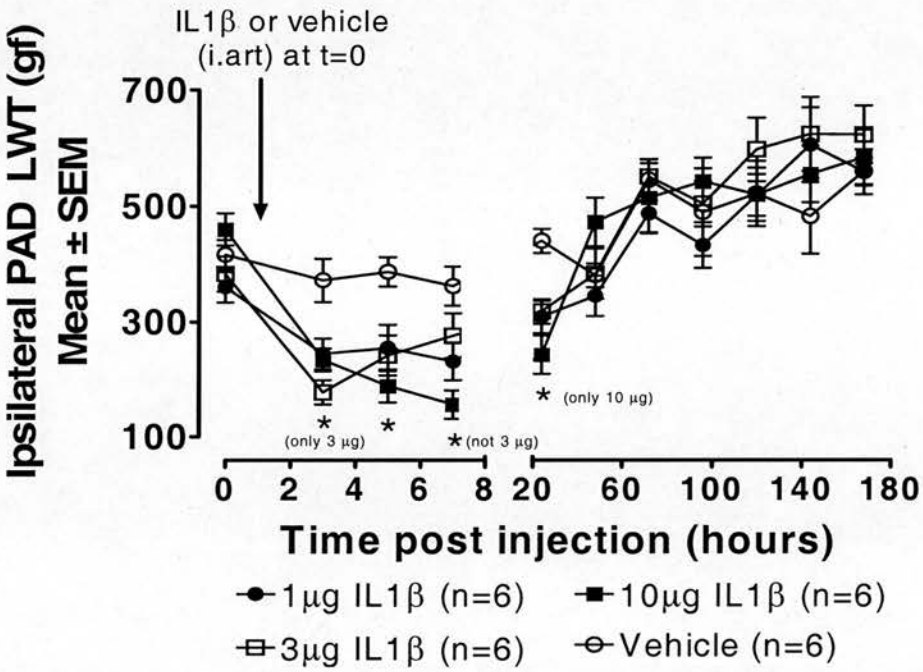


Figure 6.6 Ipsilateral LWTs of rats injected with 1, 3 or 10 μg IL1β (n=6 at each dose) or vehicle (n=6; sterile saline; at t=0) following baseline measurements. The highest dose of IL1β administered evoked the longest duration of mechanical hypersensitivity (as measured by PAD); the ipsilateral LWT was significantly reduced in comparison with vehicle-treated rats until one day post-IL1β (* $P<0.05$, two-way ANOVA).

Time (hours)	Drug administered i.art into ipsilateral knee joint at t=0			
	Vehicle (n=6)	1 μg IL1β (n=6)	3 μg IL1β (n=6)	10 μg IL1β (n=6)
0	492.0 ± 63.6	399.7 ± 21.8	420.2 ± 29.7	405.7 ± 46.8
3	448.3 ± 32.8	390.2 ± 54.8	475.8 ± 60.4	443.3 ± 44.8
5	418.5 ± 44.3	510.0 ± 62.7	429.7 ± 45.7	500. ± 37.7
7	430.5 ± 28.7	476.8 ± 56.8	492.7 ± 47.5	623.8 ± 57.4
24	595.8 ± 43.6	533.5 ± 47.9	381.8 ± 52.6	437.7 ± 41.1
48	406.8 ± 69.8	372.3 ± 28.5	455.3 ± 39.5	653.7 ± 16.7
72	429.2 ± 55.3	408.8 ± 72.4	512.3 ± 41.0	707.0 ± 51.9
96	67.5 ± 49.3	410.3 ± 33.6	474.5 ± 26.9	491.8 ± 62.1
120	455.2 ± 93.3	479.2 ± 82.9	624.7 ± 46.9	632.7 ± 46.0
144	406.5 ± 63.1	604.7 ± 57.7	586.7 ± 90.7	632.0 ± 93.5
168	473.2 ± 36.4	710.3 ± 49.2	627.2 ± 64.4	537.5 ± 71.7

Table 6.1. Contralateral LWTs for IL1β- (1, 3 and 10 μg; n=6 at each dose) and vehicle-injected (n=6; sterile saline) rats. No significant difference in contralateral LWTs occurred between IL1β-injected and vehicle-treated animals at any time during this study ($P>0.05$, two-way ANOVA). The grey box indicates the pre-IL1β or vehicle baseline values.

6.3.5 EFFECT OF IL1β ON ACUTE CYTOKINE RELEASE IN THE JOINT SPACE

The acute (up to seven hours) effects of IL1β (1 μg; n=6) on the production of inflammatory mediators in the joints was investigated and compared with vehicle-injected joints (n=6). IL1β significantly increased the expression of IL1β, IL6 and TNFα in ipsilateral joint perfusates in comparison with levels in vehicle-injected joints ($P<0.05$, two-way ANOVA, see Figure 6.7).

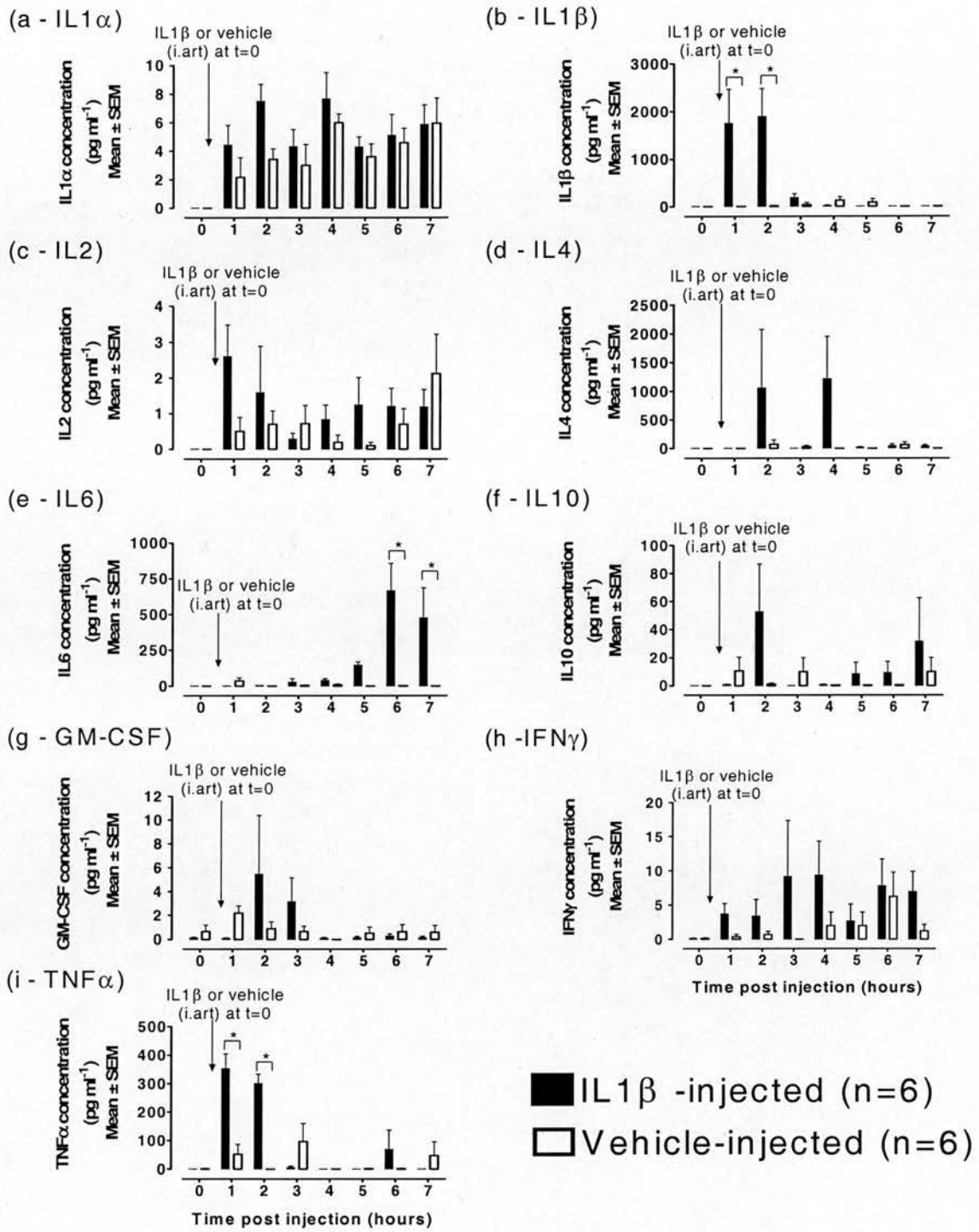


Figure 6.7 Levels of (a) IL1α, (b) IL1β, (c) IL2, (d) IL4, (e) IL6, (f) IL10, (g) GM-CSF, (h) IFNγ and (i) TNFα in normal joints (t=0) perfused with IL1β (1 μg; n=6; closed bars) or vehicle (sterile saline; n=6; open bars) and sampled hourly until seven hours post-administration. IL1β caused significant increases in IL1β, IL6 and TNFα production in comparison with vehicle-injected joints (* $P<0.05$ two-way ANOVA).

6.3.6 EFFECT OF IL1β ON "CHRONIC" CYTOKINE RELEASE AND INFLAMMATORY CELL INFILTRATION IN THE JOINT SPACE

The "chronic" (up to ten days) effects of IL1β (1 μg; n=3) on the production of inflammatory mediators in the joints was investigated and compared with vehicle-injected joints (n=3). It was found that IL1β significantly increased levels of IL1α, IL1β, IL2, IL6, IL10 and TNFα, in ipsilateral joint perfusates in comparison with levels in vehicle-injected joints ($P<0.05$, two-way ANOVA, see Figure 6.8).

The "chronic" effects of IL1β (1 μg; n =3) on inflammatory cell infiltration into the joint space was investigated and compared with vehicle-injected joints (n=3). IL1β induced a significant increase in the total number of inflammatory cells ($0.55 \pm 0.43 \times 10^6$ cell ml^{-1}) in the joint perfusate sample on day one after administration in comparison with levels in vehicle-injected joints (0 ± 0; n=3; $P<0.05$, two-way ANOVA). No cells were detectable at any other time point during this study.

6.3.7 EFFECT OF IL1β ON HISTOLOGY OF THE NORMAL JOINT

I.art IL1β resulted in a marked influx of inflammatory cell infiltrate from day one after administration, as shown in Figure 6.9. This effect decreased by day 4 and by day 7 no abnormalities were observed. At no time did IL1β (1 μg; n=3 on days 1, 2, 4 and 7) cause cartilage or bone erosion.

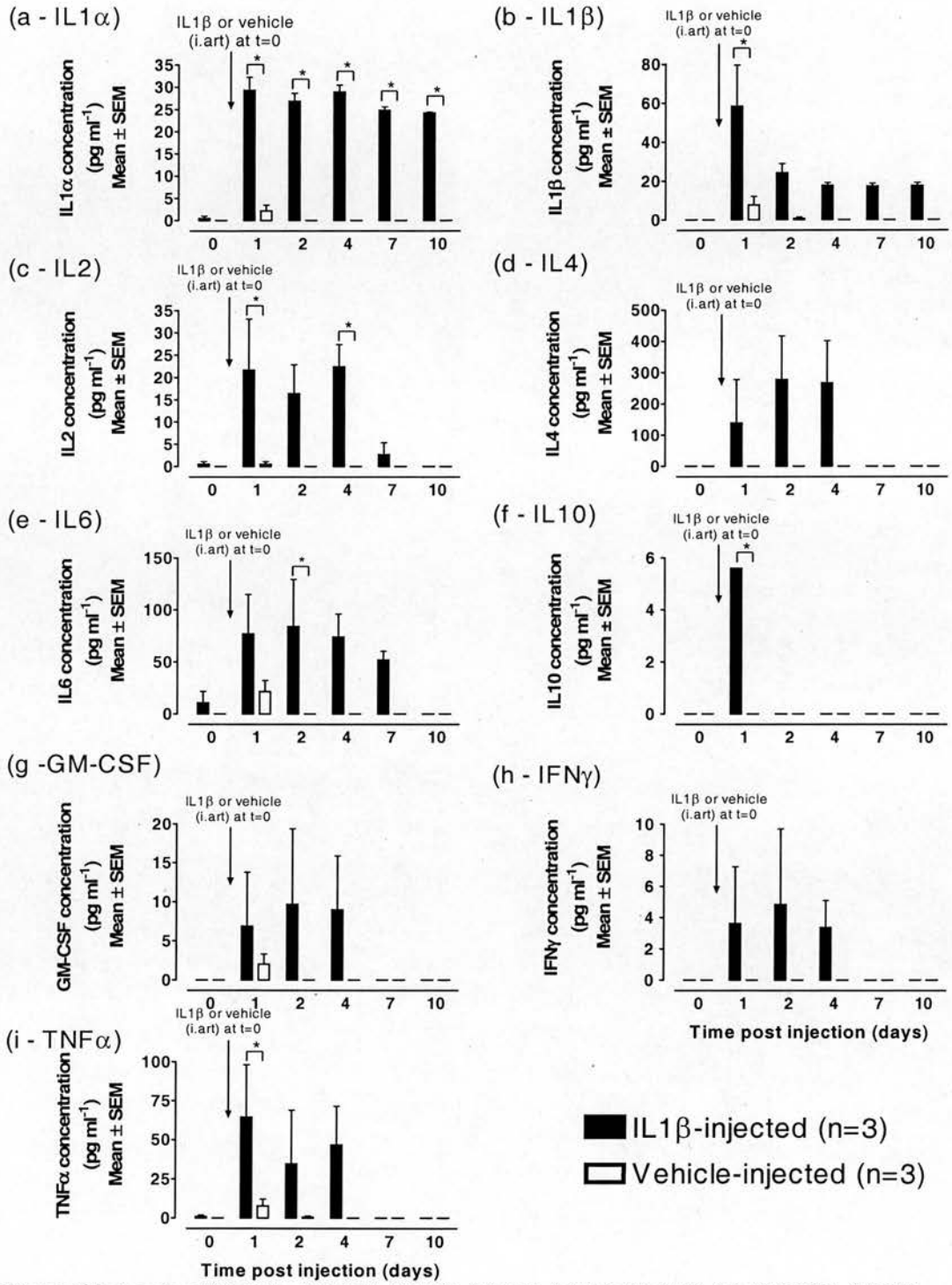


Figure 6.8 Levels of (a) IL1α, (b) IL1β, (c) IL2, (d) IL4, (e) IL6, (f) IL10, (g) GM-CSF, (h) IFNγ and (i) TNFα in normal joints (t=0) injected with IL1β (1 μg; n=3) or vehicle (100 μl sterile saline; n=3) and sampled daily until ten days post-administration. IL1β resulted in significant increases in IL1α, IL1β, IL2, IL6, IL10 and TNFα production in comparison with vehicle-injected joints (* $P<0.05$ two-way ANOVA).

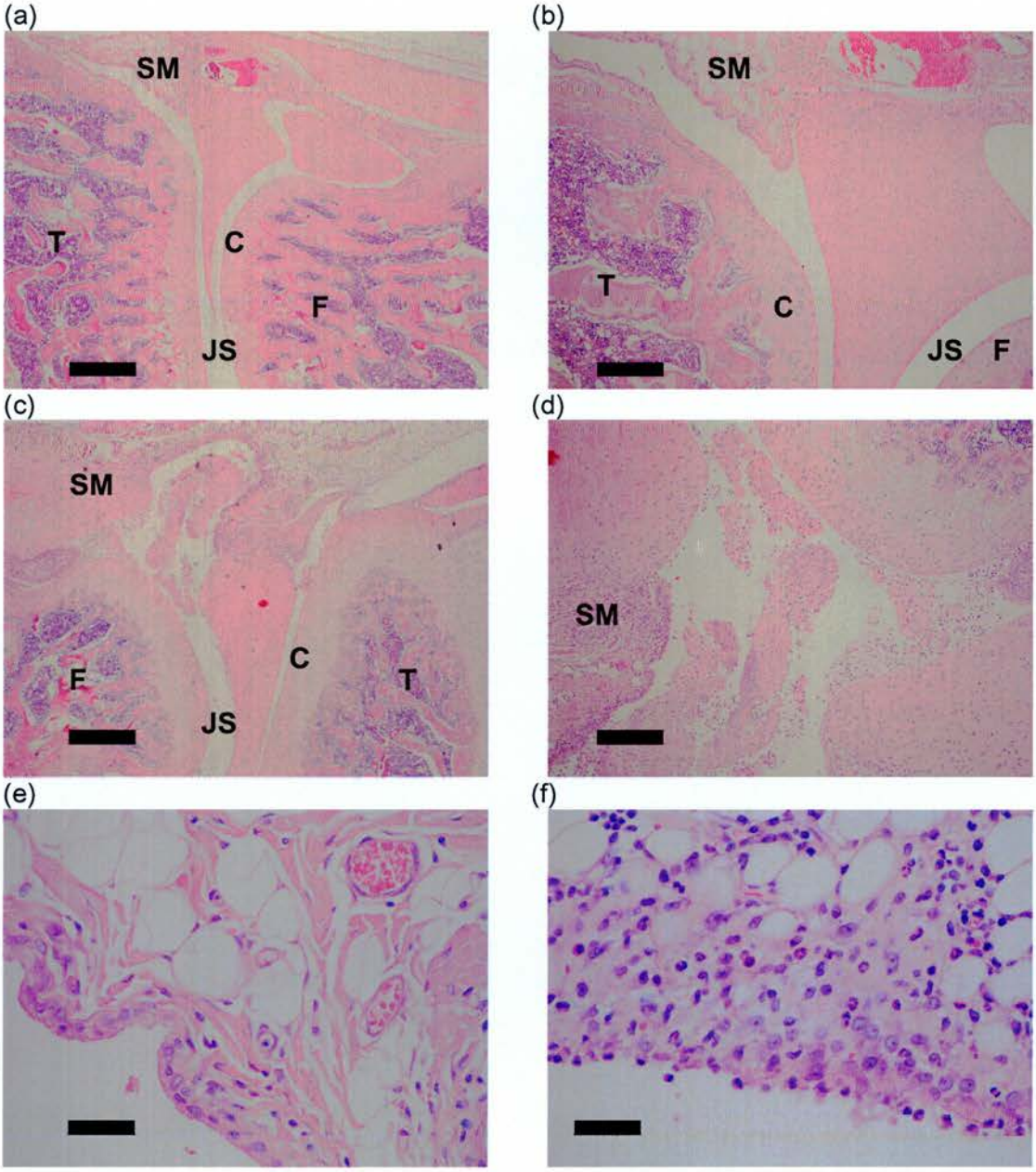


Figure 6.9 Summary of histology from (a) and (b) normal (untreated) and (c) and (d) IL1β-injected (day 1 post IL1β; 1 μg; n=3) rat knee joints respectively. The normal joint shows an acellular joint space, thin synovial membrane and smooth, healthy cartilage. (e) The normal and (f) IL1β-injected (day 1 post IL1β; 1 μg; n=3 on days 1, 2, 4 and 7) synovium of a rat knee joint. PMNs and macrophages infiltrate the joint space and synovium, although no structural changes to the joint occurred as a result of IL1β (n=3). Midline sections stained with H&E.
Abbreviations: JS, joint space; C, cartilage; T, tibia; F, femur; SM, synovial membrane.
Scale bars: (a) 500μm, (b) 250 μm, (c) 500 μm, (d) 250 μm (e and f) 50 μm.

6.4 DISCUSSION

The aims of these studies were to investigate the role of IL1β in joint pain and inflammation. The contribution made by primary afferent fibres to mechanical hypersensitivity of the joint after administration of i.art IL1β was investigated by recording action potentials from primary afferent nerves innervating the knee joint. I.art IL1β caused a transient increase in the frequency of basal neural discharge by 88% within three hours. It also decreased the threshold of mechanical stimulation required to evoke neural activity by 50% between one and four hours after injection. However, IL1β did not alter the firing frequency to mechanical stimulation above the threshold. The induction of spontaneous neural activity coincides with the occurrence of spontaneous pain during inflammation, such as that measured by the incapacitance tester as a result of i.art IL1β in the present study. The present results are similar to those of others, who report that intra-plantar injection of IL1β induced changes in sensitivities and the receptive field of DRG neurons (Fukuoka *et al.*, 1994). A reduction in the LWT following IL1β occurred within a few hours, similar to the decrease in the mechanical threshold to von Frey hairs in primary afferents, probably as a result of neuronal sensitisation.

IL1β AND PGs

IL1β is a potent nociceptive agent (Ferreira *et al.*, 1988) that induces the release of PGs (Dayer *et al.*, 1979; Handwerker & Neher, 1976; Mizel *et al.*, 1981) which sensitise nociceptors in humans and animals (Ferreira, 1972; Schaible & Schmidt, 1988). This is probably the way IL1β acts in the present study, given that the effects were not immediate, although there may be direct stimulatory action on certain sensory nerves (Follenfant *et al.*, 1989). Evidence also suggests that IL1β may increase the release of peptides from peptidergic neurons. It increased the Substance P-like immunoreactivity in the culture medium of primary cultured rat DRG cells (Inoue *et al.*, 1999). The latter effect was calcium dependent and significantly inhibited by

IL1Ra, COX inhibitors and dexamethasone (Inoue *et al.*, 1999). The roles of PGs, BK, Substance P, or other mediators could be investigated by giving enzyme inhibitors of these compounds, or receptor antagonists during the present study design. A further investigation which may prove interesting, to determine if the effects of IL1β on sensory nerves is dose-dependent, would be to increase the doses of IL1β administered during the electrophysiological recordings. This was not carried out during this thesis due to financial and time limitations.

IL1β AND HYPERSENSITIVITY

Although IL1β did not cause swelling of the joint, it did induce mechanical hypersensitivity within a couple of hours, which lasted for up to four days. Previously, i.art IL1β has been shown to evoke swelling of the joint, although at higher doses than those used in the present study (90 μg rather than 10 μg; Chandrasekhar *et al.*, 1990). However, it is evident from the results in Chapter 5 that IL1β alone is not entirely responsible for inflammatory joint swelling. Furthermore, mice that spontaneously develop arthritis and are additionally IL1β -/- develop milder forms of arthritis than do IL1β+/+ arthritic mice, with swelling occurring in only a few digits in those mice lacking IL1β (Hata *et al.*, 2004). In addition, neutralisation of IL1β did not reduce joint swelling in mice with SCW arthritis, whereas anti-TNFα treatment did (Kuiper *et al.*, 1998). Not all rats respond to IL1β to the same degree, which may indicate differences in the number or location of the receptors between individuals, and this may explain why anti-IL1β treatment is not effective in all RA patients, and why not all inflamed joints contain IL1β (see Chapter 5).

Previous studies have provided evidence that proinflammatory cytokines induce or facilitate inflammatory as well as neuropathic pain or hyperalgesia. Direct receptor-mediated actions of cytokines on afferent nerve fibres have been reported as well as cytokine effects involving further mediators. For example, electrophysiological recording from rat DRGs during

topical application of IL1β resulted in an increase in the discharge rate, increased mechanosensitivity of the DRG units (Ozaktay, 2006). Furthermore, brief applications of IL1β to nociceptive neurons yielded a potentiation of heat-activated inwad currents and a shift of activation thresholds towards lower temperatures without altering intracellular calcium levels, the effect was not mediated by G-protein coupled receptors but was mediated by activation of protein kinases (Obreja *et al.,* 2002; for review of cytokines and pain see Sommer & Kress, 2004).

IL1β AND COX-2

IL1β stimulates the production of COX-2 and the subsequent release of PGs (Bernheim *et al.*, 1980; Crofford *et al.*, 1994; Cunha *et al.*, 1992; Zucali *et al.*, 1986). The fact that PGs ultimately sensitise joint nociceptors suggests IL1β as an important inflammatory hypernociceptive mediator. In fact, IL1β was the first cytokine reported to mediate inflammatory nociception in experimental animals (Ferreira *et al.*, 1988). Ferreira *et al* (1988) reported that IL1β, even at picomolar doses, produced a severe mechanical hypernociception that apparently depends on the release of prostanoids, as local pre-treatment with indomethacin blocked its effects. The peripheral pro-nociceptive action of IL1β is mediated by a complex signalling cascade and secondary production of NO, BK or PGs (for review see Poole *et al.*, 1999). However, IL1β can excite nociceptive fibres in the DRG *in vivo* within one minute of plantar administration of IL1β between 100 pg and 1 μg, indicating a more direct action (Fukuoka *et al.*, 1994). IL1β applied to nociceptors in the skin-nerve preparation resulted in a facilitation of heat-evoked release of CGRP from peptidergic neurons (Opree & Kress, 2000), indicating that IL1β may play a important role in thermal hyperalgesia during inflammation or injury.

IL1β AND JOINT EROSION

Inflammation and skeletal destruction in arthritis are determined by interactions between numerous pro- and anti-inflammatory molecules. IL1β and TNFα are two key players that work co-operatively to promote joint inflammation. IL1β and TNFα exhibit synergism *in vitro* when inducing production of PGE_2 (Berenbaum *et al.*, 1996; Meyer *et al.*, 1990), IL6 (Harigai *et al.*, 1991), or IL8 (Rathanaswami *et al.*, 1993) by human synovial fibroblasts. IL1β and TNFα also act synergistically to recruit leukocytes into rabbit joints *in vivo* (Henderson & Pettipher, 1989). It has been proposed that synergism between these two cytokines results from reciprocal positive feedback cycles by which IL1β induces TNFα production (Ikejima *et al.*, 1990) and *vice versa* (Turner *et al.*, 1989); IL1β has also been shown to induce expression of itself (Schindler *et al.*, 1990). In the present study IL1β had no effect on bone or cartilage, which is in contrast to other studies which found that both IL1β and TNFα yielded mild inflammation, and evoked significant bone resorption, osteoclasts and cartilage matrix loss (Bolon *et al.*, 2004; Campagnuolo *et al.*, 2003). However, the dose used in the present study was at the lower end of those used by others; although it did induce functional signs of inflammation, further cytokine release and changes in the primary afferent activity. In rabbits, IL1β (5 μg; i.art) failed to cause significant proteoglycan loss from cartilage, although it did induce inflammatory cell recruitment (Henderson & Pettipher, 1989), in accord with our results.

IL1β AND CYTOKINE RELEASE

IL1β-evoked mechanical hypersensitivity that was accompanied by an increase in the expression of IL1β, IL6 and TNFα within the first eight hours, and additionally with elevated levels of IL1α, IL2 and IL10 from 24 hours post-IL1β. Increases in TNFα were observed within an hour of IL1β administration, whereas IL6 expression was delayed until six hours after IL1β injection. IL1β induced IL2 receptor up-regulation and production of IL2 *in vitro*, and also

induced IL6 mRNA expression (Panzer *et al.*, 1993). In the present study, IL1β induced significant IL2 protein production within a day of administration, and although it didn't achieve statistical significance, due to variation, there was an increase in IL2 protein in the acute studies within one hour of IL1β dosing.

CONCLUSIONS

In conclusion, the present studies have demonstrated that i.art IL1β can evoke mechanical hypersensitivity via actions on afferents innervating the knee joint and may be at least partly responsible for spontaneous pain via actions on peripheral nerves. This could be confirmed using anti-IL1β treatments, such as Anakinra, a recombinant version of endogenous IL1Ra, following IL1β administration in the above studies. Moreover the role of PGs, BK, Substance P, 5-HT, ATP, NO, or other mediators in IL1β-mediated pain could also be proven using COX, BK, substance P, NO and 5-HT inhibitors, although time and financial constraints prevented these studies for the purpose of this thesis. However, it is unlikely that IL1β is solely responsible for joint pain and inflammation, for example during RA. IL1β evoked the release of a number of pro- and anti-inflammatory cytokines within a few hours of injection into the knee joint. However, it must be noted that in comparison to the physiological levels of cytokines measured in rat knee joints during adjuvant-induced arthritis, the doses administered in the studies reported in this chapter are pharmacological, therefore not potentially exaggerating the role of inflammatory protein on the parameters measured. Anti-IL1β treatments, such as Anakinra are now used to treat patients with RA, and although they have proved promising in reducing overt signs of inflammation and pain, and slowing the progression of the disease and joint damage, there is a proportion of patients who do not respond. In addition, those who do respond do not see total resolution of their symptoms. Therefore, a combination of anti-cytokine drugs may be more efficacious, as mentioned in Chapter 5.

Chapter 7 – The role of IL6 in joint pain and inflammation

7.1 INTRODUCTION

IL6 is one of the most abundant cytokines found in both the joints and blood of patients with active RA, for more information see Section 1.4. It is produced in synovial joints by lymphocytes, monocytes, fibroblasts (Kishimoto, 1992), synoviocytes (Guerne *et al.*, 1989) and endothelial cells (Hirano *et al.*, 1988). IL6 acts synergistically with IL1 and TNFα to augment the production and release of MMPs and increase the production of tissue inhibitors of MMP from human synovial fibroblasts (Ito *et al.*, 1992). Therefore IL6 may modulate the balance between MMPs and inhibitors of MMPs at sites of inflammation in RA; it is this imbalance that may result in joint and cartilage destruction. IL6 is also produced by osteoblasts and chondrocytes and is induced by pro-inflammatory cytokines (Guerne *et al.*, 1990; Hierl *et al.*, 1998; Littlewood *et al.*, 1991). In bone, IL6 is an autocrine and paracrine factor that plays a role in osteoclast-mediated bone resorption (Ishimi *et al.*, 1990; Roodman *et al.*, 1992). However, IL6 does not appear to have a direct effect in stimulating bone resorption mediated by mature osteoclasts (Bertolini *et al.*, 1994). In addition, IL6 is responsible for:

- B-cell growth and differentiation
- Stimulating B-cell differentiation to plasma cells (Hirano *et al.*, 1986), which may account for the high levels of rheumatoid factor in RA patients.
- Stimulating proliferation and differentiation of T lymphocytes into cytotoxic T cells (Okada *et al.*, 1988)
- Promoting leukocyte chemotaxis (Taga & Kishimoto, 1997), by inducing the expression of ICAM-1 and other adhesive ligands.

IL6 knockout mice are protected against joint inflammation and destruction in both CIA and antigen-induced arthritis (Alonzi *et al.*, 1998; de Hooge *et al.*, 2000; Ohshima *et al.*, 1998). This protection was seen despite the expression of both TNFα and IL1 in the inflamed synovium

(Ohshima *et al.*, 1998), demonstrating the importance of IL6 in these models of disease. Furthermore, an IL6 receptor- neutralising antibody suppressed the onset and reduced the severity of CIA in mice (Takagi *et al.*, 1998). IL6 blockade using a monoclonal antibody was well-tolerated and resulted in significant improvement in clinical scores and laboratory parameters in a small number of patients with RA (Wendling *et al.*, 1993). A recent therapeutic trial of an anti-IL6 receptor monoclonal antibody in patients with active RA resulted in a significant, dose-dependent reduction in disease activity (Choy *et al.*, 2002).

However, there is also evidence that IL6 has anti-inflammatory effects. It can reduce inflammation by suppressing IL1 and TNFα production and by inducing the release of IL1 Ra and soluble TNFα receptors *in vitro* (Taga & Kishimoto, 1997). Hence it has been argued that IL6 may regulate rather than mediate inflammation. In addition, zymosan-induced arthritis in IL6-/- mice showed increased cartilage degradation, which occurred in spite of reduced joint swelling and normal production of IL1, TNFα and NO (van de Loo *et al.*, 1997). IL6 may therefore have both deleterious and protective effects in inflammatory arthritis. The aims of this part of the study were:

- To determine the effects of IL6 on acute and chronic joint swelling, hypersensitivity and joint destruction
- To assess the release of other pro- and anti-inflammatory cytokines as a result of i.art IL6
- To determine the effect of IL6 on primary sensory afferents innervating the normal rat knee neural activity and sensitivity using *in vivo* electrophysiological recording.

7.2 METHODS

All methods were carried out as described in Chapter 2.

Perfusion of the knee joint in anaesthetised rats was carried out as described in Section 2.4. To determine the acute effects of IL6 on the release of pro- and anti-inflammatory cytokine release, the left knee joints of six normal (untreated) rats were perfused to collect basal samples. Then recombinant rat IL6 (1 μg in 100 μl; n=6) or vehicle (100 μl sterile saline; n=6) was perfused into the joint over one minute. One, two, three, four, five, six and seven hours later a 250 μl sample was collected via perfusion of the joint, the sample was frozen and stored at -20° C.

To evaluate the effects of IL6 on the cytokine content of the joint over a ten day period, IL6 (1 μg in 100 μl; n=15) or vehicle (100 μl sterile saline; n=15) were administered via i.art injection under transient halothane anaesthesia (3% in oxygen). Animals were allowed to recover, and the ipsilateral (injected) knee joints of three rats were perfused on days 1, 2, 4, 7 and 10 post-IL6 (n=3 per day) or vehicle (n=3 per day). Total inflammatory cell counts of joint perfusate samples were carried out as described in Section 2.4.2, samples were then frozen and stored at -20° C and later assayed for a range of nine cytokines (IL1α, IL1β, IL2, IL4, IL6, IL10, GM-CSF, IFNγ and TNFα) using the Luminex assay as described in Section 2.4.3.

7.3. RESULTS

7.3.1. EFFECT OF IL6 ON BASAL NEURAL DISCHARGE OF PRIMARY AFFERENT NERVES

The basal neural discharge frequency of primary afferents innervating normal rat knee joints was 0.01 ± 0 impulses s^{-1} (n= 13 rats, 36 units; 78 % of units were inactive at this time). This increased to 0.03 ± 0.01 impulses s^{-1} (n= 8 rats; 27 units; 33 % of units were inactive at this

time) or 0.07 ± 0.04 impulses s^{-1} (n= 5 rats; 9 units; 45 % of units were inactive at this time) 30 minutes after i.art IL6 (0.1 μg) or vehicle respectively, these values were not significantly different from each other (P>0.05, Mann Whitney). Between 30 and 180 minutes after IL6 the elevated neural discharge frequency increased towards a peak, when the frequency was 0.11 ± 0.05 impulses s^{-1} (n=8 rats, 27 units), however, this was not significantly different than that of vehicle-treated joints at this time point (P>0.05, two-way ANOVA; see Figure 7.1).

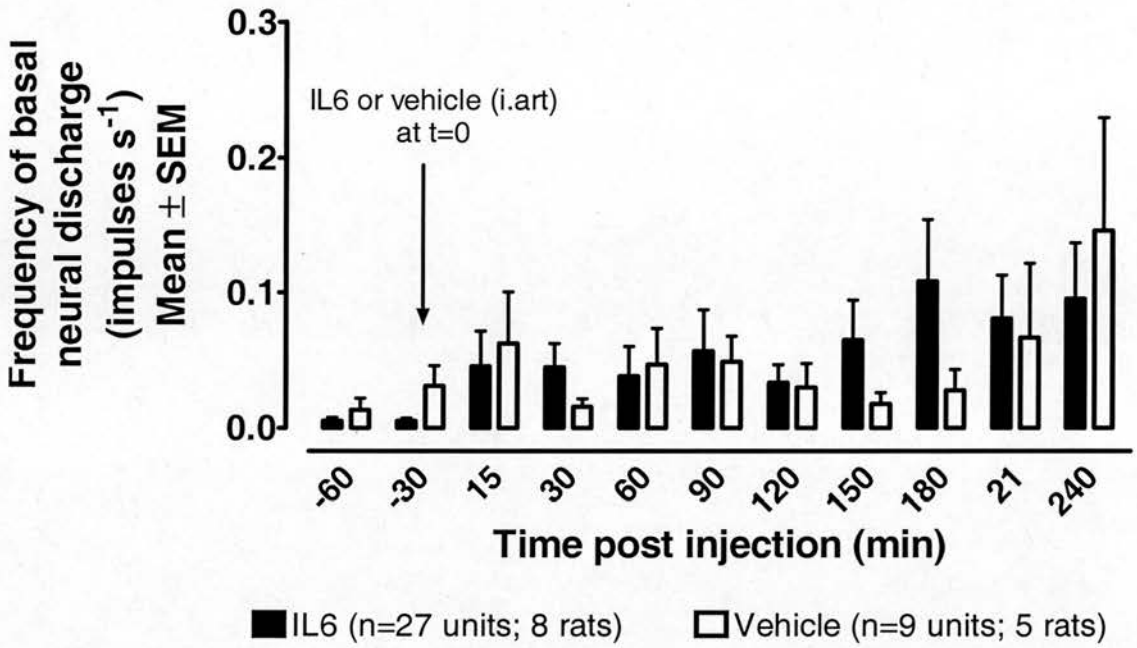


Figure 7.1 Basal neural activity (impulses s^{-1}) in fibres of the MAN in normal rat knee joints injected with IL6 (n=27 units; 8 rats; 0.1 μg; closed bar) or vehicle (n=9 units; 5 rats; open bar; sterile saline) at time t=0 (arrow). There was no significant difference between basal neural discharge frequency in IL6- and vehicle-injected joints at any time (P>0.05, two-way ANOVA)

7.3.2 EFFECT OF IL6 ON THE MECHANICAL THRESHOLD OF PRIMARY AFFERENT NERVES

The mechanical threshold for activation of primary afferent nerves innervating the normal rat knee joint was 4.99 ± 0.31 g (n=13 rats, 36 units). Following IL6 there was no significant difference in the threshold in comparison with vehicle-injected joints (P>0.05, two-

way ANOVA; see Figure 7.2). The afferents included in this analysis probably include a mixture of nociceptors and low threshold mechano-sensitive afferents.

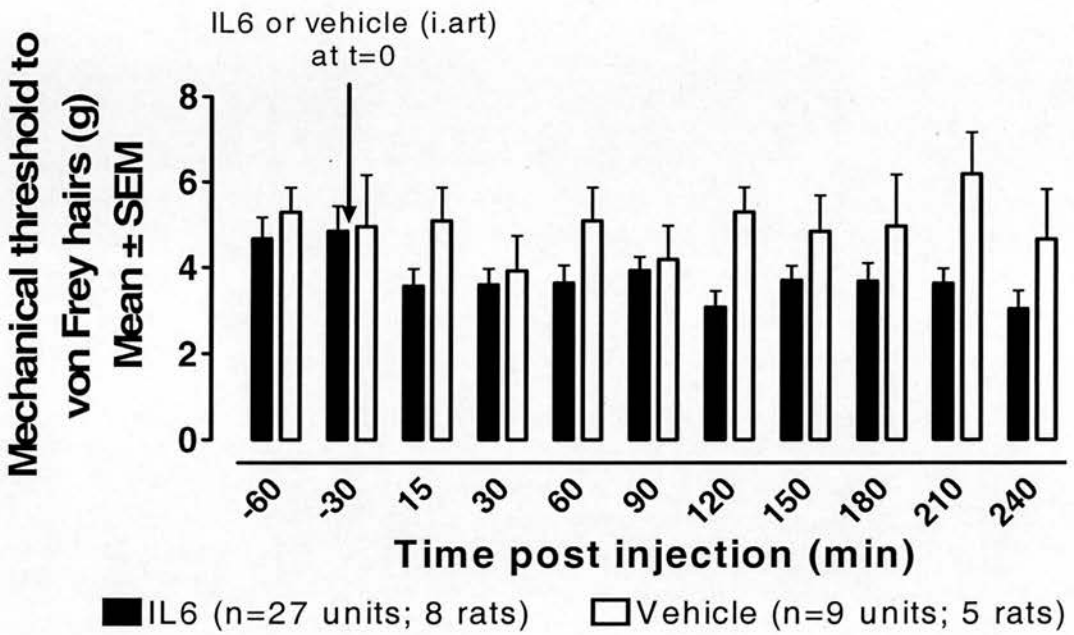


Figure 7.2 Mechanical threshold of primary afferent nerves in normal rat knee joints injected with IL6 (n=27 units; 8 rats; 0.1 μg; closed bar) or vehicle (n=9 units; 5 rats; open bar; sterile saline) at time t=0 (arrow). IL6 had no effect on the mechanical threshold to von Frey hairs in comparison with vehicle-injected joints (P>0.05, two-way ANOVA).

7.3.3 EFFECT OF IL6 ON DISCHARGE FREQUENCY AND ACTION POTENTIAL COUNT TO MECHANICAL STIMULATION

The evoked response of primary afferents innervating the normal rat knee joints to 0.98, 12.5 and 20.9 g von Frey hairs were 0.12 ± 0.08, 3.34 ± 1.31 and 5.98 ± 2.34 impulses s^{-1} respectively (n=12 rats; 35 units). Following IL6 (0.1 μg; n=6) or vehicle (n=6), no significant changes to the evoked discharge frequency or total action potential count occurred (P>0.05, two-way ANOVA; see Figure 7.3).

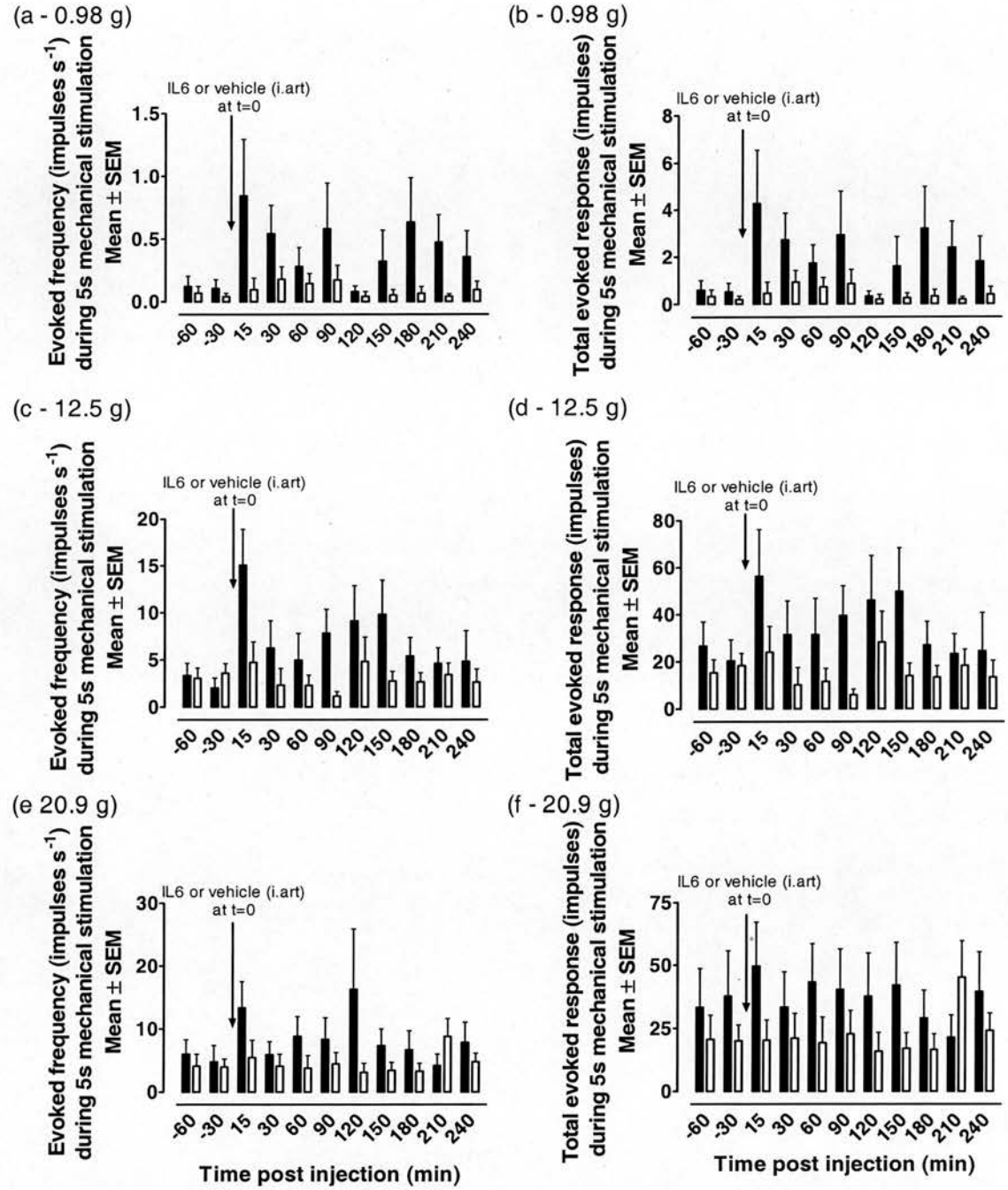


Figure 7.3 The evoked frequency and total action potential count to five seconds of mechanical stimulation in the receptive field of a primary afferent innervating normal rat knee joints. Stimulation with (a) and (b) 0.98 g, (c) and (d) 12.5 g and (e) and (f) 20.9 g von Frey hairs in normal joints and following IL6 (n=20 units; 6 rats; 0.1 μg; closed bar) or vehicle (n=15 units; 6 rats,; open bar; sterile saline) at time t=0. IL6 had no effect on the evoked discharge frequency to mechanical stimulation by these von Frey hairs in comparison with vehicle-injected joints (P>0.05, two-way ANOVA, Bonferroni post hoc test).

7.3.4 EFFECT OF IL6 ON JOINT SWELLING AND MECHANICAL HYPERSENSITIVITY

I.art IL6 had no adverse effects on the animals' general health as demonstrated by the fact that animals continued to feed and gain weight normally throughout the study (see Figure 7.4a). Three, four, five, six and seven days post-IL6 the body weights of the rats were significantly higher than day 0 ($P<0.05$, Kruskal Wallis). There was no significant difference in body weight between the IL6 and vehicle-injected animals at any time point ($P>0.05$, 2-way ANOVA).

Joint swelling

Before the i.art injection, the ipsilateral and contralateral knee joint diameters were 9.1 ± 0.1 mm (n=24) and 9.1 ± 0.0 mm (n=24) respectively, which were not significantly different ($P>0.05$, Mann Whitney). Following IL6 (1, 3 or 10 μg; n=6 at each dose) there was no significant difference in joint diameter in comparison with those of vehicle-injected joints (n=6) at any time point ($P>0.05$, two-way ANOVA; see Figure 7.4b). No changes in contralateral joint diameters occurred in comparison to the basal values at any time during this study ($P>0.05$, Kruskal Wallis).

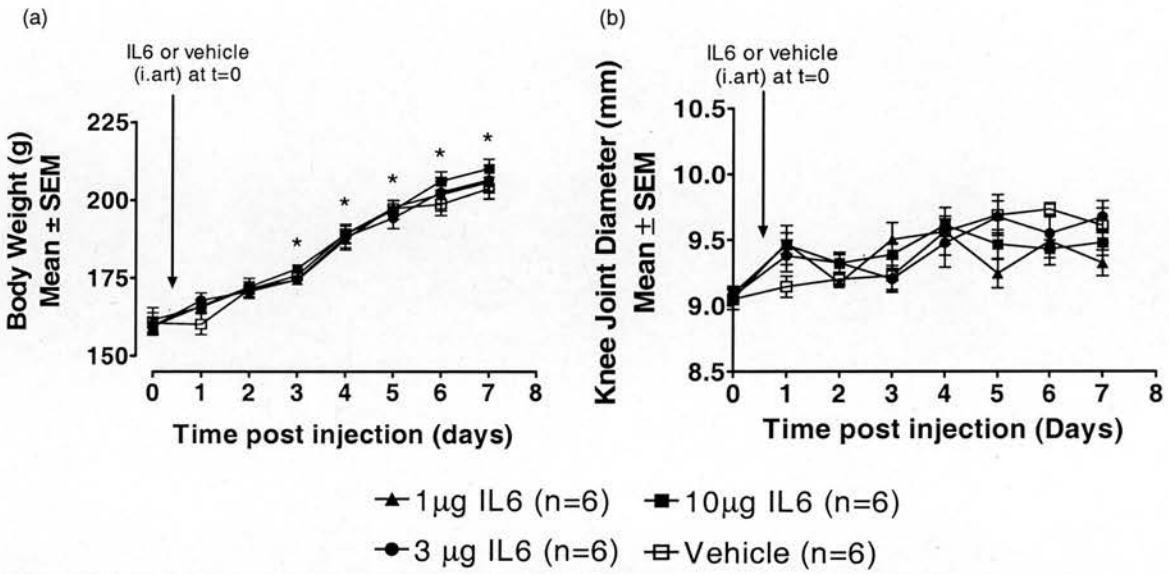


Figure 7.4. The (a) body weight and (b) knee joint diameters of IL6- and vehicle-injected rats over a seven day study. IL6 (1, 3 or 10 μg; n=6) had no effect on knee joint diameter in comparison with vehicle-injected (sterile saline) rats at any time point during this study (n=6; * P>0.05, two-way ANOVA). Statistical analysis was performed to compare the body weight or joint diameter of the two groups on each day using the two-way ANOVA and a Bonferroni post-hoc test.

Mechanical hypersensitivity measured by the incapacitance tester

Prior to injection of IL6, the weights on the two hind limbs were 52.4 ± 2.3 g (n=24) and 51.3 ± 1.78 g (n=24), with an associated ratio (ipsi:contra) of 1.1 ± 0.0. However, following administration of 1, 3 and 10 μg IL6 the weights on the ipsilateral (injected) and contralateral (uninjected) hind limbs were significantly different from each other until 48 hours post-injection (P<0.05, Kruskal Wallis; see Figure 7.5 a, b and c). The ratio of the weight distribution between the hind limbs decreased by 57.4 ± 0.04% (n=6), 69.9 ± 0.1% (n=6) and 51.6 ± 0.1 % (n=6) in rats treated with 1, 3 and 10 μg IL6 respectively by four hours post administration. The ratio remained significantly reduced in comparison with vehicle-injected joints (n=6) until day one post-IL6 (P<0.05, two-way ANOVA; see Figure 7.5d).

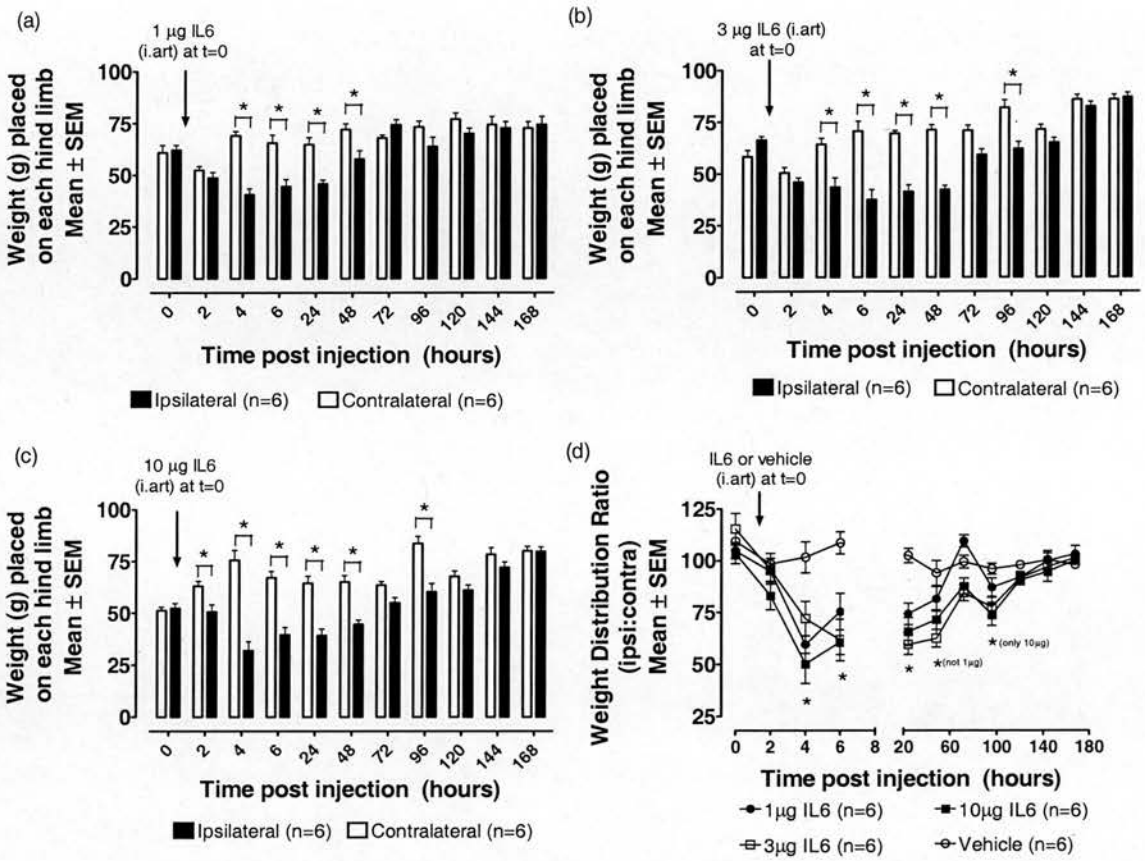


Figure 7.5 The absolute weight placed on the ipsilateral (injected) and contralateral hind limbs of rats injected with (a) 1 μg, (b) 3 μg and (c) 10 μg IL6 (n=6) on day 0 after basal measurements were made and (d) the associated ratio of weight distribution between the hind limbs for IL6- and vehicle-injected (sterile saline) rats. All doses of IL6 caused significant mechanical hypersensitivity of the joint from two hours post administration (* $P<0.05$, two-way ANOVA). Statistical analysis was performed to determine whether the difference between the weight on the ipsilateral and contralateral hind limbs were significantly different (a, b, and c) or whether the ratio of weight distribution was significantly reduced in IL6-injected rats compared with vehicle-injected animals using a two-way ANOVA.

Mechanical hypersensitivity measured by PAD

Before injection of IL6, the ipsilateral and contralateral LWTs were 415.8 ± 22.1 gf (n=24) and 468.5 ± 49.5 gf (n=24) respectively, which were not significantly different ($P>0.05$, Kruskal Wallis; n=6). However, following IL6 (10 μg; n=6) the ipsilateral LWT was reduced between five until seven hours post-IL6 in comparison with vehicle-injected rats (n=6; $P<0.05$, two-way ANOVA; see Figure 7.6). No changes in the LWT of contralateral joints were observed at any time during this study ($P>0.05$, two-way ANOVA, see Table 7.1).

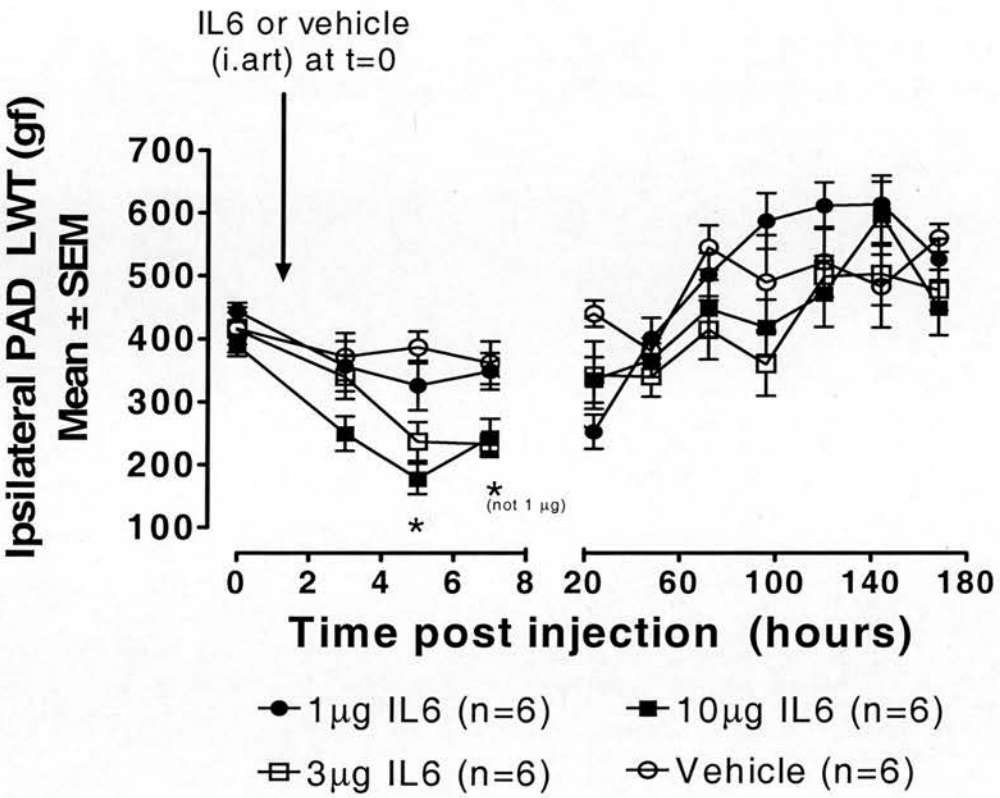


Figure 7.6 Ipsilateral LWTs for rats injected with 1, 3 or 10 μg IL6 (n=6 at each dose) or vehicle (n=6; sterile saline; at t=0) following baseline measurements. IL6 (3 ans 10 μg) evoked a significant decrease in LWT from five until seven hours post-IL6 in comparison with vehicle-treated rats (* $P<0.05$, two-way ANOVA).

Time (hours)	Drug administered i.art into the ipsilateral knee joint at t=0			
	Vehicle (n=6)	1 ug IL6 (n=6)	3 μg IL6 (n=6)	10 μg IL6 (n=6)
0	492.0 ± 63.6	525.8 ± 48.9	396.2 ± 41.7	461.7 ± 44.0
3	448.3 ± 32.8	409.7 ± 51.4	497.0 ± 29.9	398.0 ± 45.3
5	418.5 ± 44.3	494.3 ± 28.0	423.7 ± 45.2	422.7 ± 43.0
7	430.5 ± 28.7	474.3 ± 70.7	397.8 ± 19.2	384.3 ± 36.1
24	595.8 ± 43.6	503.2 ± 28.4	441.7 ± 35.9	524.0 ± 55.0
48	406.8 ± 69.8	474.3 ± 27.4	343.2 ± 29.5	386.7 ± 32.2
72	429.2 ± 55.3	555.2 ± 80.3	483.7 ± 43.5	408.2 ± 49.6
96	67.5 ± 49.3	468.2 ± 27.7	355.5 ± 32.0	521.3 ± 10.0
120	455.2 ± 93.3	441.7 ± 68.3	486.7 ± 58.8	392.7 ± 69.6
144	406.5 ± 63.1	660.7 ± 36.8	552.0 ± 67.3	646.2 ± 146.9
168	473.2 ± 36.4	533.8 ± 57.0	582.0 ± 55.0	533.2 ± 73.3

Table 7.1. Contralateral LWTs for IL6- (1, 3 and 10 μg; n=6 at each dose) and vehicle-injected (sterile saline; n=6) rats. No significant difference in contralateral LWTs occurred between IL6-injected and vehicle-treated animals at any time during this study ($P>0.05$, two-way ANOVA). The grey box indicates the pre-IL1β or vehicle baseline values.

7.3.5 EFFECT OF IL6 ON ACUTE CYTOKINE RELEASE IN THE JOINT SPACE

The acute (up to seven hours) effects of IL6 (1 μg; n=6) on the production of inflammatory mediators in the joints was investigated and compared with vehicle-injected joints (n=6). IL6 significantly increased the expression of IL1β, IL6 and TNFα in ipsilateral joint perfusates in comparison with levels in vehicle-injected joints ($P<0.05$, two-way ANOVA, see Figure 7.7).

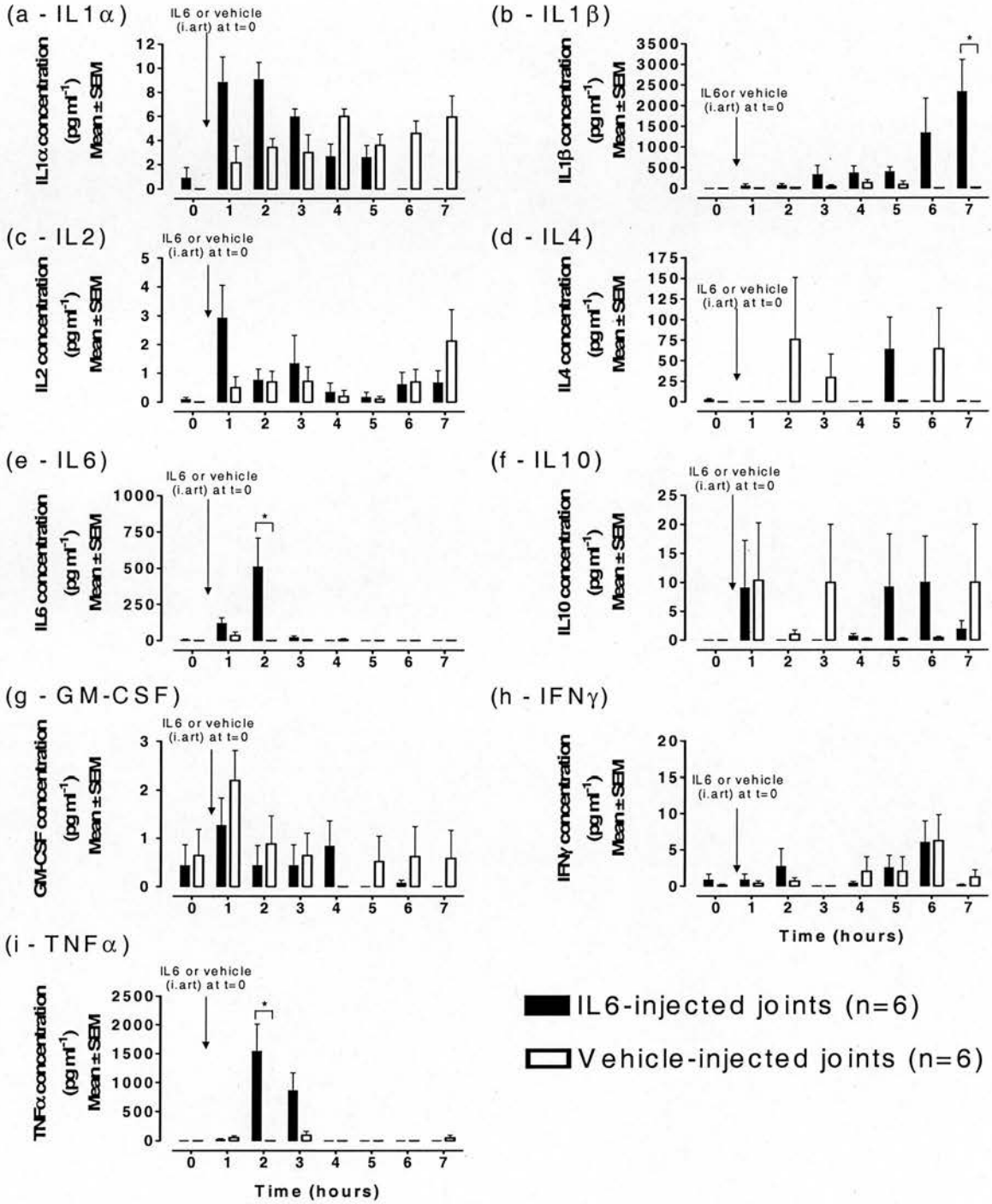


Figure 7.7 Levels of (a) IL1α, (b) IL1β, (c) IL2, (d) IL4, (e) IL6, (f) IL10, (g) GM-CSF, (h) IFNγ and (i) TNFα in normal joints (t=0) perfused with IL6 (1 μg; closed bars; n=6) or vehicle (sterile saline; open bars; n=6) and sampled hourly for seven hours post administration. IL6 caused significant increases in IL1β, IL6 and TNFα production in comparison with vehicle-injected joints (* $P<0.05$ two-way ANOVA).

7.3.6 EFFECT OF IL6 ON "CHRONIC" CYTOKINE RELEASE AND INFLAMMATORY CELL INFILTRATION IN THE JOINT SPACE

The "chronic" (up to ten days) effects of IL6 (1 μg; n=3) on the production of inflammatory mediators in the joints was investigated and compared with vehicle-injected joints (n=3). It was found that IL6 significantly increased levels IL1α, IL2, IL4 and IL6 in ipsilateral joint perfusates in comparison with levels in vehicle-injected joints ($P<0.05$, two-way ANOVA, see Figure 7.8).

The "chronic" effects of IL6 (1 μg; n=3) on inflammatory cell infiltration into the joint space was investigated and compared with vehicle-injected joints (n=3). IL6 had no effect on the total number of cells in the joint perfusate sample in comparison with levels in contralateral joints ($P>0.05$, two-way ANOVA). No cells were detectable at any time during this study.

7.3.7 EFFECT OF IL6 ON HISTOLOGY OF THE NORMAL JOINT

I.art IL6 (1 μg; n=3 on days 1, 2, 4 and 7) resulted in no detectable cellular infiltrate, cartilage or bone erosion at any time point during this study (days 1, 2, 4 or 7).

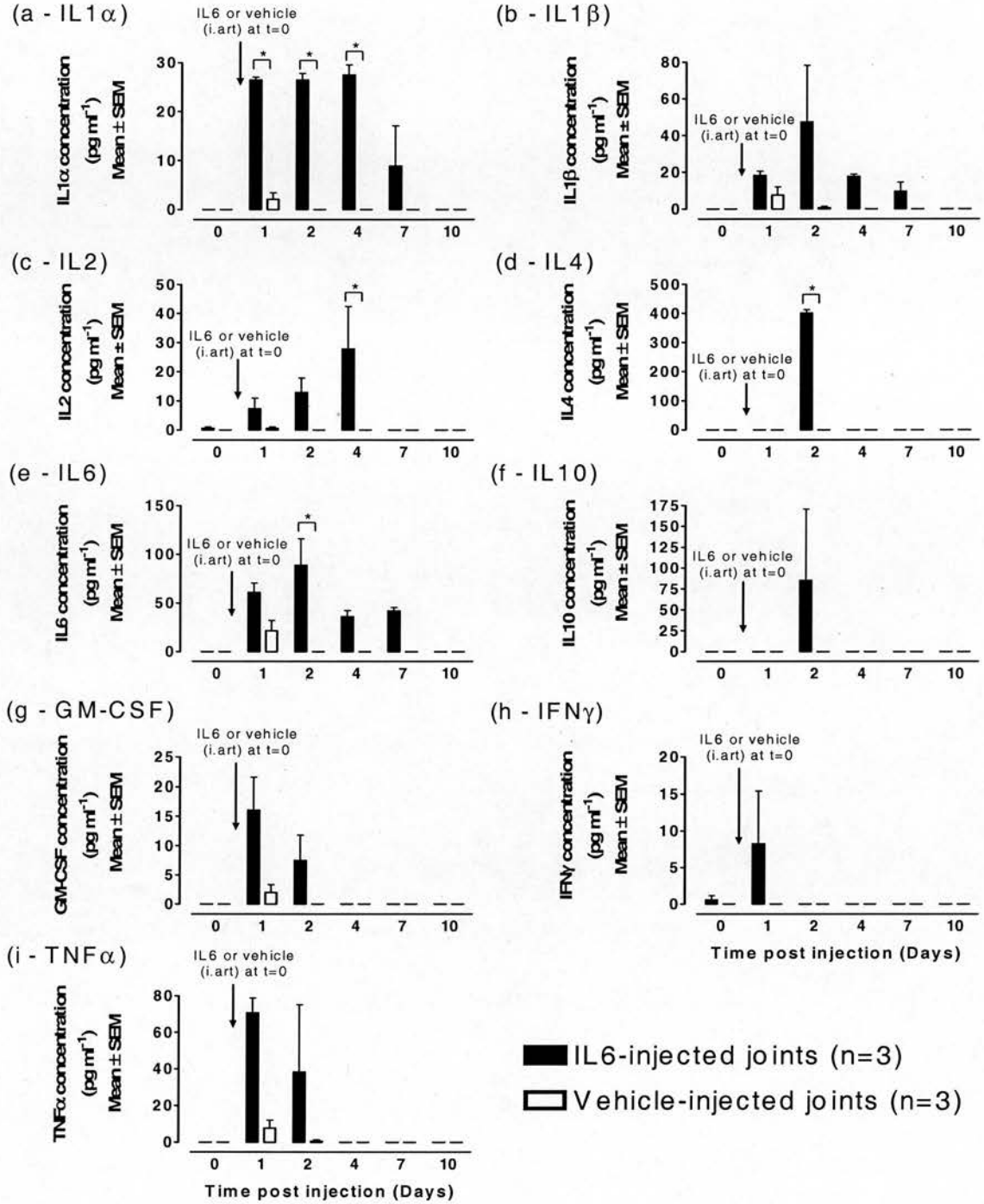


Figure 7.8 Levels of (a) IL1α, (b) IL1β, (c) IL2, (d) IL4, (e) IL6, (f) IL10, (g) GM-CSF, (h) IFNγ and (i) TNFα in normal joints (t=0) injected with IL6 (1 μg; n=3) or vehicle (sterile saline; 100 μl; n=3) and sampled daily until ten days post-administration. IL6 resulted in significant increases in IL1α, IL2, IL4 and IL6 production in comparison with vehicle-injected joints (* P<0.05, two-way ANOVA).

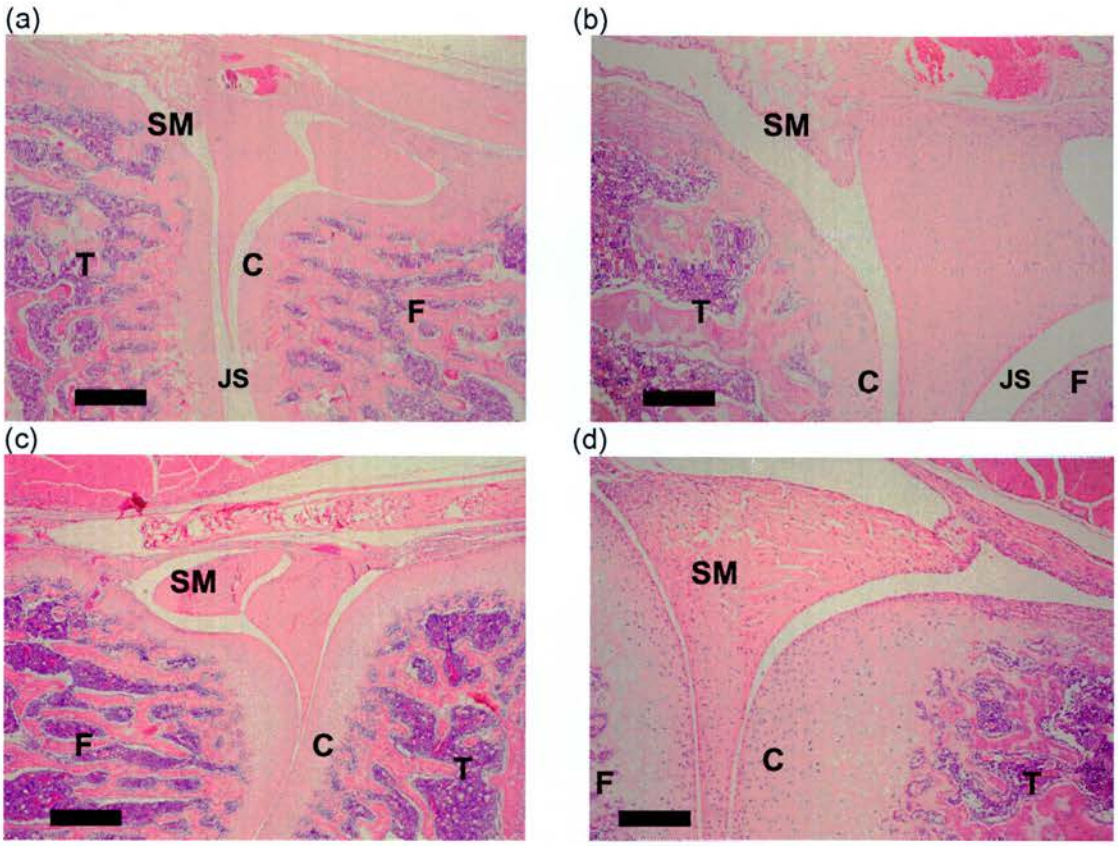


Figure 7.9 Summary of histology from (a) and (b) normal (untreated) and (c) and (d) IL6-injected (day 1 post IL6; 1 μg; n=3) rat knee joints. No changes to the joint occurred as a result of IL6 (1 μg; n=3 on days 1,2, 4 and 7). Midline sections stained with H&E.
Abbreviations: JS, joint space; C, cartilage; T, tibia; F, femur; S, synovial membrane; SM, synovial membrane
Scale bars: (a) 500μm, (b) 250μm, (c) 500μm, (d) 250μm.

7.4 DISCUSSION

IL6 had no significant effect on the activity or sensitivity of primary afferent nerve fibres innervating the normal rat knee joint at the dose used in this study. It did not affect the frequency of basal neural discharge, the mechanical threshold, or the frequency of neural discharge evoked by mechanical stimulation above the threshold for activation. Although IL6 (0.1 μg) had no effect on the activity of peripheral primary afferents, it did induce mechanical hypersensitivity of the joint when injected at higher doses (1, 3 and 10 μg). Different doses

were used in the two studies, as the electrophysiological study was carried out first, in which doses were selected based on existing literature. However, when the behavioural study was designed an existing behavioural study (unpublished pilot study from our laboratory) and current literature indicated that 0.1 μg IL6 had no effect on swelling of hypersensitivity of the joint, therefore higher doses were chosen.

IL6 AND HYPERSENSITIVITY

In accord with our results, injection of IL6 in a rat hind paw was reported to induce a dose-dependent mechanical hyperalgesia in both hind paws, although this was greatest in the ipsilateral paw (Cunha *et al.*, 1992). This mechano-allodynia, which was maximal with 1 ng of human IL6, reached a plateau between 2 and 3 h after injection, persisted for at least 6 h, and returned to pre-injection values within 24 h. The dose used in the latter study was lower than the doses used in the present study, although this may reflect differences between the paw and the knee joint, or with the different measures of mechanical hypersensitivity used, von Frey hairs, a punctuate stimulus rather than the more widespread stimulus applied using PAD in the present study. In a recent study, using the same preparation as that described in this theis, Brenn *et al.* (2007) demonstrated that IL6 has the potential to sensitize c-fibres in the joint to mechanical stimulation, therefore suggesting that IL6 contributes to mechanical hypersensitivity due to an action on nerve fibres themselves. The latter study administered a coinjection of sIL^R, which may explain the differing results.

Evidence in the literature for a precise role of IL6 is confusing since there have been pro- and anti-inflammatory effects demonstrated. It has also been reported that intracerebroventricular injections of IL6 induced thermal hyperalgesia in naïve rats (Oka *et al.*, 1995), whereas intracisternal IL6 had anti-nociceptive effects in an acute orofacial pain model. However, intra-plantar IL6 had no effect on thermal withdrawal latencies between uninjured rats

(DeLeo *et al.*, 1996), although it increased mechanical thresholds of inflamed rat hind paws (Czlonkowski *et al.*, 1993). Studies using IL6 knockout mice have not been conclusive in establishing the role of endogenous IL6 in nociception. Two studies found no difference in thermal and mechanical withdrawal latencies of IL6 deficient mice and wild type mice (Bianchi *et al.*, 1999; Murphy *et al.*, 1999), whilst others report increased thermal thresholds in IL6 -/- mice (Zhong *et al.*, 1999) or decreased mechanical and thermal thresholds (Xu *et al.*, 1997). The reasons for the different outcomes of these studies are unclear, although the knockout of IL6 appears to result in compensatory effects such as a threefold increase in TNFα levels (Fattori *et al.*, 1994).

Moreover, a direct effect of IL6 on the neural activity of DRG cells in rats was not found by Ozaktay *et al.* (2006), which is similar to the results in this thesis, although electrophysiological recording during my studies was from a peripheral primary afferent nerve, this may be in part due to the lack of sIL6R administered in both studies. The IL6/sIL6R complex acts agonistically on cells that express the signal transducer molecule gp130. Previous *in vitro* and *in vivo* studies demonstrate that short exposure to the IL6/sIL6R complex modulates nociceptors specific release of CGRP thus raising the possibility that IL6 complexed with the sIL6R coul directly sensitize the nociceptors to noxious stimuli (Obreja, O *et al.,* 2002; Opree & Kress, 2000).

IL6 AND INJURY

Peripheral nerve-like structures exhibit IL6-like immunoreactivity in normal human skin and to a greater extent in inflamed skin (Nordlind & Vahlquist, 1999). Furthermore, nerve injury results in increased levels of IL6 mRNA or IL6-positive cells in rat and mouse sciatic nerves (Bourde *et al.*, 1996; Cui *et al.*, 2000; Reichert *et al.*, 1996; Zhong & Heumann, 1995).

However, despite the large amount of literature describing the up-regulation of IL6 following nerve injury, data on the functional effects of IL6 in sensory processing is limited.

CLINICAL USE OF ANTI-IL6 TREATMENTS

Clinically, five patients treated with a murine anti-IL6 monoclonal antibody (i.v.; 10 mg day^{-1}) daily for 10 consecutive days showed rapid clinical improvement in pain, morning stiffness, the number of tender and swollen joints, and reduction in CRP (Wendling *et al.*, 1993). A recombinant humanised antimonoclonal antibody of the IgG1 subtype (MRA) was tested in a phase I/II double blind, randomised, placebo controlled, single dose trial in patients with RA. The ACR20 response criterion was met by 50% of patients in the 5mg kg^{-1} group at week two and none in the placebo group. Improvement was maintained until week eight. A significant difference in ACR 20 was also noted between the group receiving 10 mg kg^{-1} MRA and that receiving placebo at weeks six and eight (Choy *et al.*, 2002).

IL6 AND JOINT EROSION

As IL6 is known to activate osteoclasts, it has been suggested that it plays a role in the joint erosion that occurs during RA. However, no effects on bone or cartilage were detected in the present study, although only one dose of IL6 was studied in an attempt to adhere to the Home Office's 3Rs and due to financial and time constraints. In contrast, IL6 has been proposed to have a protective effect on cartilage (Shingu *et al.*, 1995; van de Loo *et al.*, 1997; van den Berg, 1999), therefore i.art injection of IL6 would not be expected to cause joint damage. However, an alternative approach to determine if IL6 is involved in joint erosions would be to treat FCA-injected animals with anti-IL6 drugs and monitor the joint erosions before, during and after IL6-directed therapy.

CONCLUSIONS

In conclusion, results from these studies suggest that IL6 is able to produce mechanical hypersensitivity of the joint, although was not able to induce swelling, joint damage or alterations in sensitivities of primary afferent fibres. Further work that would add significantly to this information would include increasing the doses of IL6 administered during electrophysiological recordings, and co-administration of IL6 receptors in any of the studies described in this chapter, to investigate whether IL6 was not effective due to the lack of receptors in the normal joint. Studies similar to those described here but carried out in FCA-injected rats may have different outcomes if the IL6 R is unregulated during inflammation. In addition, it must be noted that in comparison to the physiological levels of cytokines measured in rat knee joints during adjuvant-induced arthritis, the doses administered in the studies reported in this chapter are pharmacological, therefore not potentially exaggerating the role of inflammatory protein on the parameters measured.

It should be emphasised that IL6 is not the only molecule that modulates nociceptive pathways. All members of the "IL6 cytokine family" share the transmembrane signal transducer, gp130, resulting in a degree of redundancy (Ito *et al.*, 1998). In addition, other mediators and cytokines influence pain. Tissue injury elicited by trauma or surgery brings about immediate, well localised pain. This pain is sustained after the initial injury, implying that substances are produced to maintain pain. IL-6 is produced in substantial quantities at the site of a surgical wound (Holzheimer & Steinmetz, 2000), giving an indication that IL6 may well play a dominant role in the development of pain and inflammation during an immune response.

Chapter 8 – Discussion and Future Directions

The aims of this thesis were to investigate the temporal expression pattern of inflammatory mediators and cells in the knee joints of rats with adjuvant-induced arthritis, and to determine the roles of IL1β and IL6 in initiating and maintaining joint pain and inflammation using two novel techniques alongside existing methods.

8.1 DEVELOPMENT OF NOVEL TECHNIQUES

Two novel techniques were developed and validated during this thesis to improve the study of experimental inflammation and to aid screening of novel drugs designed to treat RA in humans.

A new behavioural readout which was designed to assess localised, mechanical hypersensitivity of rodent knee joints was developed. PAD uses similar principles to the clinical pressure dolorimeter (Langley *et al.*, 1983). The method involved the application of a gradually increasing squeeze directly across the knee joint until the rodent shows signs of pain or discomfort, as evidenced by withdrawal of the limb, or rarely vocalisation. At this "end point" a quantitative value of the maximum force applied prior to limb withdrawal is recorded, and this value provided an objective LWT. PAD provides a reliable, quantitative measurement of localised, FCA-induced mechanical hypersensitivity in the knee joint of rats and mice observed as a decrease in LWTs of around 40% and 60% respectively, compared with the basal values in normal joints, which were approximately 1045 gf in rats and 400 gf in mice. Moreover, PAD was able to detect the analgesic action of prednisolone in both species, in addition to analgesia induced by morphine and celecoxib in rats. A strong correlation was observed between the weight distribution readout and the PAD measurements in both species, illustrating that PAD is a simple behavioural test that will be valuable for assessing hypersensitivity in rodent joints with the particular advantage of assessing the hypersensitivity at the site of the inflammation, in a similar fashion to the clinical dolorimeter and in contrast to the incapacitance tester.

The second new technique was designed to collect the "inflammatory soup" within the joint cavity for subsequent analysis of inflammatory mediator and cell content. This method was validated by a series of experiments investigating

- whether the experimental protocol resulted in any changes to the cytokine content of the joint
- whether the method could be used to detect differences between normal and inflamed knee joints
- whether samples collected via perfusion of the joint contained detectable levels of inflammatory cells.

It was established that the choice of anaesthetic may play a role in initiating an acute inflammatory response within the knee joint. It was decided to use urethane for joint perfusion experiments, based on the results in Section 4.3.1. In addition, it was observed that some animals showed increased levels of TNFα or IL1β as a result of prolonged (seven hour) placement of the perfusion needles in the joint, but the changes occurred in only 20% of animals and were not statistically significant. Furthermore, very little, if any, solution perfused into the joint is lost into the surrounding tissue, and the perfusate can all be recovered through the effusion tubes. Combined, this data indicates that the perfusion experimental set up does not evoke inflammatory reactions, and that the system is efficient, with very little leakage from the joint cavity.

FCA triggered a significant increase in levels of TNFα and IL1β in the inflamed (ipsilateral) joint. In addition, a biphasic expression pattern of PGE_2 following FCA was observed during a 21 day FCA time course, as well as the possibility that PGE_2 is present in normal joints, although further studies would be required to prove this was not an artefact of the needle insertion, as described in Chapter 4. Not surprisingly it was found that FCA-injected joints contained higher levels of inflammatory cells than normal rat knee joints, however, it was

also noted that contralateral joints contained measurable levels of inflammatory cells, despite the fact that no overt signs of swelling or hypersensitivity are evident.

This perfusion technique proved to be reliable and consistent; samples were easily collected from chronically inflamed joints, and no problems were experienced whilst measuring the cytokine or cell content of the perfusate samples. This technique is therefore a valuable addition to protocols which use homogenates of entire joints to assess inflammatory mediator content.

FUTURE DIRECTIONS

PAD could be further validated by using it to assess mechanical hypersensitivity of the knee joint during other models of inflammatory joint pain, or alternatively for assessing hypersensitivity in other regions of the body, such as the paw or ankle joints. PAD could be used as a mechanical stimulator of the knee joint or paw during electrophysiological recording, either from peripheral afferents, as described in this thesis, or from DRG neurons used elsewhere (Stanfa & Dickenson, 2004; Urch & Dickenson, 2003). If PAD was applied to measure hypersensitivity of the paw it could be validated against von Frey thresholds or the Randall and Selitto analgesymeter (Randall & Selitto, 1957). However, the surface area of the application pad may require adjustment, as the paw has a smaller surface area than the knee joint and may be able to withstand higher pressures, i.e. have a higher noxious threshold, in which case a transducer with a larger force range may be required.

The joint perfusion method could be used for a variety of applications; any mediator (peptides, other cytokines, chemokines or prostaglandins) within the joint space could be measured in perfusate samples during FCA-induced arthritis, as well as any other joint inflammation models, such as CIA or SCW arthritis. In addition, the effectiveness of anti-

inflammatory, anti-rheumatic or analgesic drugs on these parameters could be assessed, for example NSAIDs, anti-inflammatory drugs, DMARDs and anti-cytokine agents.

The technique was used here to measure the total inflammatory cell count, encompassing a range of inflammatory cells, probably including macrophages, lymphocytes and neutrophils. Perfusate samples could be processed to allow identification of specific cell types, for example using flow cytometry. Flow cytometry is a method for quantifying components or structural features of cells primarily by optical means. Since different cell types can be distinguished by structural features, flow cytometry can be used to count cells of different types in a mixture (Berner *et al.*, 2000).

The joint perfusion system was applied to here to measure the inflammatory mediator and cell content of rat joints, although it may be possible to adapt it for use in other species, including mice. However, the considerably smaller scale of the knee joint may make this difficult, but smaller needles and more precise placement may make this feat possible. This technique could be applied to knockout mice lacking specific inflammatory proteins, mediators or receptors and be used to determine whether downstream processes are affected by this knockout and to what extent the changes affect the inflammatory response. This would provide valuable information on the inflammatory cascade and give an indication of further potential therapeutic targets.

Both techniques could be applied in the future to improve the current level of knowledge of the inflammatory processes in the knee joint and the effectiveness of novel compounds. During this thesis, they were used to define the temporal expression patterns of a range of pro- and anti-inflammatory cytokines over a three week time course of adjuvant induced arthritis in rats, and to determine the effects of IL1β and IL6 on the expression of other pro- and anti-inflammatory cytokines as well as the influx of immune cells into the joint. In the long term, this technique could be modified to allow repeated sampling from individual rats, with the

implantation of an indwelling catheter in the joint that would allow perfusion of the joint under transient anaesthesia. However, such a device must not cause local inflammation, pain or discomfort to the rats, or allow infections into the joint. Even further down the line measurement of inflammatory mediators and cells inside the joint may not require such invasive procedures. The use of modern imaging with immunofluoresence or radiolabelled inflammatory mediators may allow identification of specific inflammatory cells or proteins in awake rats without the need for invasive surgery or procedures. However, current technology and costs make this unfeasible at this time.

8.2 CYTOKINE EXPRESSION IN INFLAMED JOINTS

Cytokine expression changed with time as FCA-induced arthritis progressed from the acute to the more "chronic" phase (14-28 days post-FCA). The levels of all nine mediators measured had substantially increased within a few days of FCA administration. The levels of some cytokines then stayed elevated, for example IL1α, but most peaked in the acute phase and then declined toward basal levels thereafter. Significant increases in levels of IL1α, IL1β, IL2 and TNFα occurred in inflamed joints. Levels of the other cytokines measured were increased in comparison with basal levels but were not statistically significant. The proportion of inflamed joints that contained detectable levels of IL1α, IL1β, IL2, IL6, IL10, GM-CSF, IFNγ and TNFα was significantly increased during the study, suggesting that the majority of joints contain the latter cytokines, although the absolute levels required to maintain joint inflammation and hypersensitivity are low, or that other mediators or mechanisms are involved in the more "chronic" phase of the model, including central mechanisms. In addition, significant correlations between measures of joint swelling with levels of IL1α, IL1β, IL4 and IL10 or between mechanical hypersensitivity with levels of IL1α, IL1β, IL6 and TNFα in inflamed joints were seen.

Prednisolone did not affect the absolute levels of cytokines in inflamed joints, although it reduced the proportion of FCA-injected joints that contained detectable levels of IL1α, IL6 and TNFα whilst significantly reducing the joint swelling over the dosing period. There appeared to be two populations, "responders" to prednisolone and "non-responders" in terms of cytokine expression, although all animals dosed with prednisolone showed reduced joint swelling in comparison to non-drug treated rats. However, in prednisolone "non-responders" the levels of cytokines measured did not change from pre-treatment levels, despite the reduction in the joint diameter measurement, indicating that prednisolone has an all-or-none effect on cytokines in this study.

As well as showing additional information regarding the expression patterns of cytokines during adjuvant-induced arthritis, close similarity between this animal model of joint inflammation and human RA have been demonstrated, which endorse the use of this model for studying the inflammatory process of human RA, and should also improve the confidence in the model to facilitate preclinical screening of novel compounds, thereby enhancing translation from laboratory to clinic during the drug development process.

CLINICAL IMPLICATIONS

The fact that there was a proportion of inflamed animals that did not express some of the key pro-inflammatory cytokines during adjuvant-induced arthritis indicates that the inflammatory response may vary between rats. It may therefore be the case in humans, and hence certain anti-cytokine treatments for RA are not effective in all patients (Cohen *et al.*, 2004; Maini *et al.*, 2006; Smolen & Steiner, 2003), and in those that they are effective, 100% improvement in the condition is not seen. Compensatory mechanisms may also be responsible for the latter effects. Anti-TNFα agents, such as etanercept or infliximab show ACR20 responses in 76% (Spencer-Green, 2000) and 53% (Zhang *et al.*, 2006) of RA patients

respectively; anti-IL1β treatments are effective in approximately 40-50% of patients (Bresnihan, 2001; Cohen *et al.*, 2004) and Tocilizumab, an anti-IL6 treatment, shows ACR 20 responses in approximately 60% of RA patients (Maini *et al.*, 2006). However, when the improvement level is raised to the ACR 50 or 70 mark, the number of responders decreases in all three treatment groups.

Combination therapy with IL-1Ra and soluble TNFα receptor I in rats with CIA resulted in an additive effect on clinical and histological parameters when moderate to high efficacious doses of each protein were administered (Bendele *et al.*, 2000). However, greater-than-additive effects were seen when an inactive dose of IL-1Ra was given in combination with moderately to minimally active doses of soluble TNFα receptor I. (Bendele *et al.*, 2000), indicating that a combination approach may prove to have greater effects than any biological agent alone and that lower doses than either drug alone could be used, thus reducing the cost and potential side effects of the treatment. Similar studies also show a synergism between other anti-TNFα and anti-IL1 treatments (Zwerina *et al.*, 2004).

LIMITATIONS OF THE DATA

Only the cytokines that were present within the synovial cavity were assayed in the present study. The levels of IL1β and TNFα were not significantly elevated in comparison with normal joints after one week of adjuvant-induced arthritis, which is perhaps reflecting a change in the distribution of these inflammatory proteins within the joint cavity and surrounding tissue. Furthermore, IL2, IL4, IL6, IL10 and IFNγ were never significantly increased above basal (pre-FCA) levels, which is in contrast to other studies in which entire joint homogenates or the synovial membranes alone have been assessed (Bush *et al.*, 2001; Schmidt-Weber *et al.*, 1999; Szekanecz *et al.*, 2000). It is therefore difficult to conclude that IL1β and TNFα are not required

in the "chronic" phases to maintain adjuvant-induced arthritis, merely that they are not present in the joint cavity during the "chronic" phase in the present study.

Differential cell counts or flow cytometry were not used to assess what proportion of the total inflammatory cell population belonged to which cell type. If this was carried out it may give an indication of which cells could be targeted to treat inflammatory joint disease. In addition, cells were only detectable on days 7, 10 and 21, indicating that the method is not sensitive enough to detect inflammatory cells in the perfusates when the levels are low, due to the fact that the perfusate samples were too dilute. This would be improved by collecting a smaller, more concentrated sample for cell counting, although a smaller volume means fewer analyses can be carried out, and perhaps cells, multiple cytokines and PGs could not be measured in the same samples. These studies were not performed during this thesis as a result of time and financial restrictions. The later studies in this thesis acted as merely pilot studies, in an attempt to reduce the Home Office 3 R's. If more time had been available, certain aspects of these studies would have been taken further and the n numbers increased to make the data more conclusive.

Ideally, the FCA time course could be conducted by repeated daily sampling from the same animals under transient anaesthesia, which would reduce the number of animals needed to conduct a study such as that detailed in Chapter 5. It would also make statistical comparisons over time more powerful as data from the same individual animals could be compared day to day. Moreover, AUC values could be calculated for individual animals over a drug-dosing period which would improve the statistical power of analysis carried out to determine if the drug affected the cytokine content of joints. However, repeated sampling was not possible during this thesis as a permanent indwelling catheter or similar device would be required to be surgically implanted in the rats' knee joints to prevent repeated insertion of needles and therefore repeated tissue damage and further inflammatory responses. Furthermore, this may lead to infection and

contamination from the daily sampling; hence at this stage in the development of the technique, once the knee joint was perfused, the rat was killed.

FUTURE DIRECTIONS

The time course of cytokine release in FCA-injected joints could be repeated with an alternative inflammatory stimulus and compared with adjuvant arthritis as well as human RA. A range of nine cytokines was measured during these studies, based on commercially available kits; however, there is a vast number of cytokines and other mediators, including peptides, chemokines and PGs that could be assayed in these samples. Ideally higher n values would be used for the prednisolone treatment study, which may result in more detailed analysis of the effects of the drugs and a greater statistical power, improving the ability to draw conclusions from the study, given that it was noted there were two populations, "responders" and "non-responders", which was not anticipated when the study was designed. Increasing the n numbers will help gain a better insight into the mechanisms of action of prednisolone and its effectiveness at reducing the inflammatory mediator content of inflamed joints. Administration of prednisolone directly into the knee may be successful at reducing the cytokine content of joints at the dose used, and it would be interesting to compare the results of such experiments with those from s.c. administration used in the present study. In addition, other anti-inflammatory, anti-rheumatic, or analgesic drugs could be administered to determine whether they had effects on the cytokine expression patterns of inflamed joints similar to that seen in the clinic, which was not the case for prednisolone in this thesis.

An extension of the present study would be to assess the inflammatory mediator content of whole homogenised joints, and compare the results with the data from the perfusate samples. This would establish whether a higher proportion of cytokines is evident in the surrounding

tissues of the joint, rather than in the joint cavity itself, particularly in the "chronic" phases when levels in the joint cavity appeared to be low.

The effect of prednisolone on levels of cytokines in the knee joint was investigated in chapter 5, however, it would also be interesting to evaluate the effects of the drug on the levels of cytokines in the blood, which was not done here to reduce the number of samples required to assay using the Luminex machine due to the high cost of the kits required to analyse a 96-well plate. This would establish whether the anti-inflammatory effects of prednisolone are via systemic reduction of pro-inflammatory mediators, rather than locally within the joint.

8.3 THE ROLES OF IL1β AND IL6 IN JOINT PAIN AND INFLAMMATION

Equal doses of IL1β and IL6 had different effects on the sensitivities and activities of primary afferent nerves innervating the normal rat knee joint. IL1β increased basal discharge and sensitised the nerve, resulting in a decrease in the mechanical force required to evoke neural activity when stimulated within the receptive field of the nerve. In contrast, IL6 had no effects on mechanical threshold or the basal neural activity. When administered at higher doses (1, 3 and 10 μg), both cytokines induced gross mechanical hypersensitivity measured by PAD and the incapacitance tester, although the response evoked by IL6 was shorter in duration than that caused by IL1β. There was also a difference between the two readouts of mechanical hypersensitivity used in this study. PAD and the incapacitance tester both detected hypersensitivity, although that measured by PAD was less well sustained than that measured by the weight distribution, despite the fact that the two measures correlated well in Chapter 3. This may be due to the milder nature of joint hypersensitivity evoked by IL1β and IL6 in comparison with FCA (used in chapter 3). In contrast to previous reports, IL1β did not cause bone or cartilage erosion, the formation of pannus or any other structural changes to the joint, although histology and analysis of joint perfusates showed that it evoked inflammatory cell recruitment

from day one post-IL1β administration. IL6 had no effect on the total inflammatory cell count, or the joint histology.

IL6 perfused into the joint caused the *de novo* release of IL1β and TNFα within two hours and additionally the release of IL1α, IL2 and IL4 release within a few days. However no inflammatory cells were detected at any time point post IL6 in joint perfusate samples. Administration of IL1β into the joint evoked increased expression of IL6 and TNFα within one hour and IL1α, IL2 and IL10 within one day and an influx of inflammatory cells within 24 hours of administration.

CLINICAL IMPLICATIONS

Anti-IL1β and -IL6 treatments may prove to be effective analgesics whilst simultaneously reducing inflammation. Neither cytokine alone resulted in joint swelling or joint destruction which is in agreement with other studies and data from Chapter 5, suggesting a plethora of mediators is required for the full RA phenotype, and hence inhibition of a single cytokine, although potentially beneficial, will not completely abolish symptoms or totally stop the progression of the underlying inflammatory disease.

LIMITATIONS OF THE DATA

In order to adhere to the Home Office 3Rs, although three doses of IL1β and IL6 were used during the behavioural study, only one dose was tested in electrophysiological, histology and joint perfusion experiments. Furthermore, the effect of IL1β and IL6 on the sensitivities and activity of peripheral primary afferents was tested using mechanical stimulation only. More experiments need to be performed to determine if these agents affected the threshold of neural activity to thermal or chemical stimulation. The nerve fibres that were recorded were not characterised to classify them into C- or Aδ fibres, which would improve the level of knowledge

gained from these studies. During the electrophysiological studies NSAIDs could have been administered to determine if PGs were the intermediate factor causing neural changes following IL1β. When IL1β or IL6 were given into the joint only cytokines within the synovial cavity were analysed, rather than those in the surrounding tissue, which would further our understanding of the roles of these mediators in joint pain and inflammation.

FUTURE DIRECTIONS

The sensitisation of peripheral nociceptors by cytokines during chronic inflammation of joints is an area of research that requires much more investigation. The role of the large number of mediators released during chronic inflammation in the excitation and/or inhibition of peripheral nociceptors is for the most part unknown. Much of the information that is available concerning the chemical excitation of joint afferents is extrapolated from experiments on cutaneous nociceptors or *in vitro* preparations. Detailed characterisation of joint nociceptors is necessary to determine whether they have unique pharmacological profiles or are similar to nociceptors in other regions of the body. There is a vast number of mediators that may play a role in joint hypersensitivity, all of which play a critical role in cell-cell interactions during inflammation, and as such may also influence sensory neurons. In the present study IL1β or IL6 were administered into the joint; simultaneous administration of their respective receptors in addition to the protein may increase the number of changes seen in the electrophysiological, histological and joint perfusion experiments. Administration of an NSAID during electrophysiological studies investigating the effect of IL1β on peripheral sensory neurons should reveal whether PGs are the only downstream mediators responsible for primary afferent sensitisation. If sensitisation was still evident, it would suggest that other intermediate molecules are involved in the sensitisation of nerves innervating the knee joints as a result of

cytokines and to elucidate this, antagonists of receptors known to be present on primary afferent nerve endings could be administered during the protocol use in Chapter 6 and 7.

8.4 GENERAL CONCLUSIONS

The primary conclusion that can be drawn from the data presented in this thesis is that IL1β plays a dominant role in the development and maintenance of FCA-induced joint pain and the associated oedema although to a lesser degree, as i.art IL1β alone was not sufficient to evoke swelling of the joint. The dose of IL1β administered was also not sufficient to cause joint destruction, despite resulting in an influx of inflammatory cells. The levels of IL1β protein are significantly elevated during adjuvant-induced arthritis, and showed a strong correlation to the weight distribution readout, a pre-clinical measure of hypersensitivity. In addition, intra-articular IL1β itself reduces the joint withdrawal threshold and weight carried by the inflamed limb, measures of evoked and spontaneous mechanical hypersensitivity, when measured by PAM and the weight distribution readout, respectively. Furthermore, electrophysiological recording from primary afferent nerves innervating the joint showed that i.art IL1β alters the mechanical threshold for firing and the basal rate of activity, but not the frequency of neural discharge to a suprathreshold stimulus. However, the role of IL6 still remains unclear; i.art injection of this inflammatory protein initiated a mild mechanical hypersensitivity, but did not affect neuronal responses or basal firing rates, not lead to changes to the joint structure or swelling. However, IL6 protein was present in the synovial fluid of joints from rats with FCA-induced joint inflammation and these levels were significantly related to the weight distribution results.

The use of two novel techniques has facilitated the study of cytokines, in particular the roles of IL1β and IL6 in the development of inflammatory joint pain in rodents (see Figure 8.1 for a summary). In addition, the time course of cytokine expression in the joint cavity during

adjuvant-induced arthritis has been established, which can be used in the future to assay novel anti-inflammatory or anti-cytokine agents. Moreover, direct links between mechanical hypersensitivity and IL1β and IL6 have been established, suggesting that these are key mediators involved in the development of joint pain; IL1β acts at least in part via direct/indirect effects on sensory neurons. In accord with previous work, the data presented in this thesis highlights the fact that multiple cytokines (probably in combination with other mediators) are required for long-lasting joint pain and inflammation. Therefore a combinatorial approach to treatment is required to provide the most effective treatment to the largest number of RA patients.

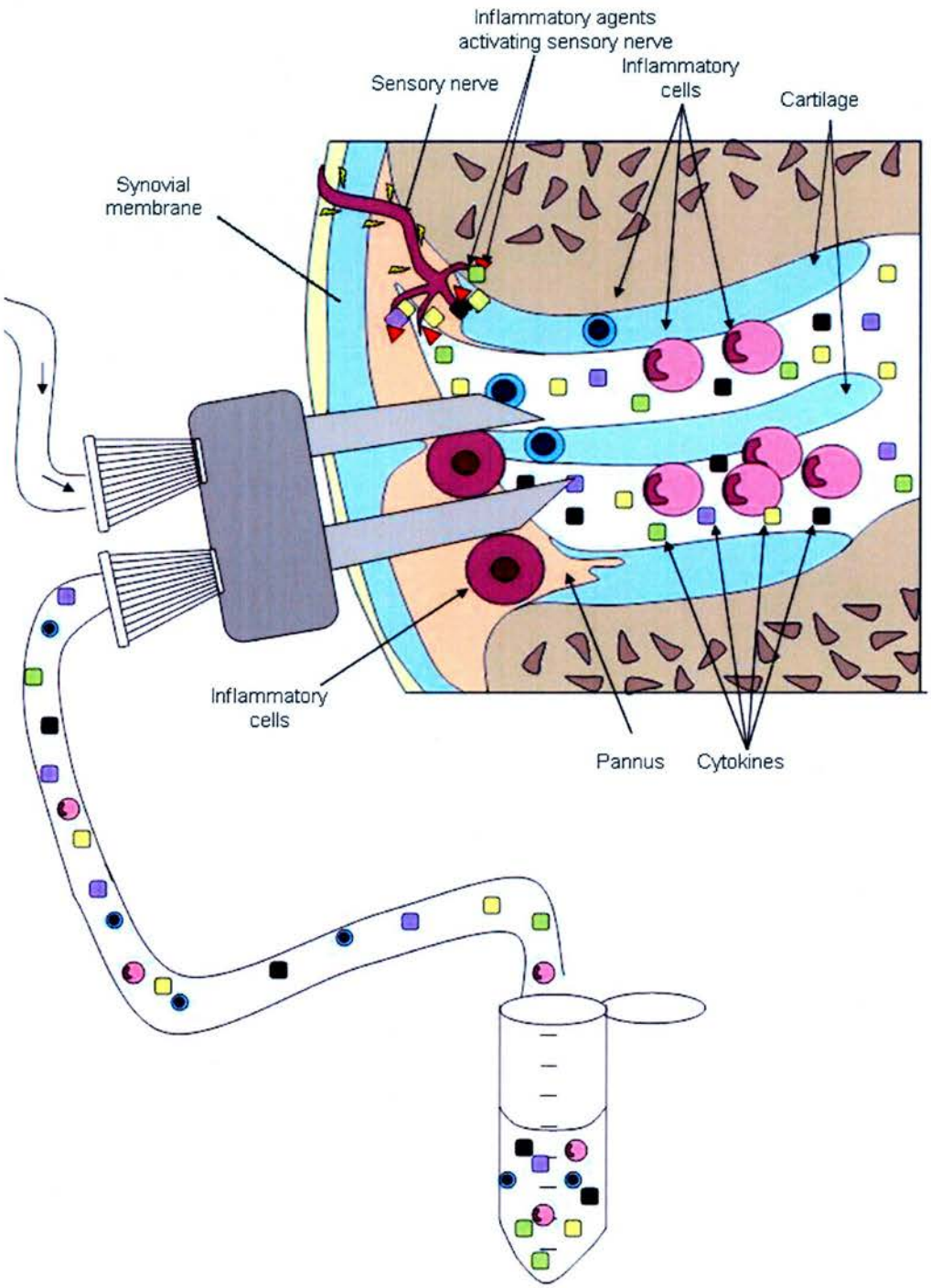


Figure 8.1 A summary of joint inflammation and the application of the novel joint perfusion technique to quantify the inflammatory mediator and cell content of the synovial cavity. This technique can be used to investigate the basic inflammatory processes and to determine the effectiveness of novel anti-inflammatory, analgesic or anti-rheumatic drugs on the levels of these mediators. A sensory nerve innervating the joint is also illustrated to indicate that inflammatory cytokines can act directly on these nerves, or indirectly via release of other pro-inflammatory and pro-nociceptive agents.

References

Aarden, L.A., De Groot, E.R., Schaap, O.L. & Lansdorp, P.M. (1987). Production of hybridoma growth factor by human monocytes. *Eur J Immunol*, **17,** 1411-6.

Abbracchio, M.P. & Burnstock, G. (1994). Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol Ther*, **64,** 445-75.

Akahoshi, T., Oppenheim, J.J. & Matsushima, K. (1988). Interleukin 1 stimulates its own receptor expression on human fibroblasts through the endogenous production of prostaglandin(s). *J Clin Invest*, **82,** 1219-24.

Akopian, A.N., Souslova, V., England, S., Okuse, K., Ogata, N., Ure, J., Smith, A., Kerr, B.J., McMahon, S.B., Boyce, S., Hill, R., Stanfa, L.C., Dickenson, A.H. & Wood, J.N. (1999). The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways. *Nat Neurosci*, **2,** 541-8.

al-Humidan, A., Ralston, S.H., Hughes, D.E., Chapman, K., Aarden, L., Russell, R.G. & Gowen, M. (1991). Interleukin-6 does not stimulate bone resorption in neonatal mouse calvariae. *J Bone Miner Res*, **6,** 3-8.

Ali, Z., Meyer, R.A. & Campbell, C. (1996). Secondary hyperalgersia to mechanical but not heat stimuli following a capsaicin injection in hairy skin. *Pain*, **68,** 401-411.

Allard, S.A., Muirden, K.D., Camplejohn, K.L. & Maini, R.N. (1987). Chondrocyte-derived cells and matrix at the rheumatoid cartilage-pannus junction identified with monoclonal antibodies. *Rheumatol Int*, **7,** 153-9.

Alldred, A. (2001). Etanercept in rheumatoid arthritis. *Expert Opin Pharmacother*, **2,** 1137-48.

Alonzi, T., Fattori, E., Lazzaro, D., Costa, P., Probert, L., Kollias, G., De Benedetti, F., Poli, V. & Ciliberto, G. (1998). Interleukin 6 is required for the development of collagen-induced arthritis. *J Exp Med*, **187,** 461-8.

Alsalameh, S., Winter, K., Al-Ward, R., Wendler, J., Kalden, J.R. & Kinne, R.W. (1999). Distribution of TNF-alpha, TNF-R55 and TNF-R75 in the rheumatoid synovial membrane: TNF receptors are localized preferentially in the lining layer; TNF-alpha is distributed mainly in the vicinity of TNF receptors in the deeper layers. *Scand J Immunol*, **49,** 278-85.

Alstergren, P., Ernberg, M., Kvarnstrom, M. & Kopp, S. (1998). Interleukin-1beta in synovial fluid from the arthritic temporomandibular joint and its relation to pain, mobility, and anterior open bite. *J Oral Maxillofac Surg*, **56,** 1059-65; discussion 1066.

Alvaro-Gracia, J.M., Zvaifler, N.J. & Firestein, G.S. (1989). Cytokines in chronic inflammatory arthritis. IV. Granulocyte/macrophage colony-stimulating factor-mediated induction of class II MHC antigen on human monocytes: a possible role in rheumatoid arthritis. *J Exp Med*, **170,** 865-75.

Anderson, D.L. (2001). Development of an instrument to measure pain in rheumatoid arthritis: Rheumatoid Arthritis Pain Scale (RAPS). *Arthritis Rheum*, **45,** 317-23.

Anderson, K.O., Bradley, L.A., Turner, R.A., Agudelo, C.A. & Pisko, E.J. (1994). Pain behavior of rheumatoid arthritis patients enrolled in experimental drug trials. *Arthritis Care And Research: The Official Journal Of The Arthritis Health Professions Association*, **7,** 64-68.

Andersson, J., Bjork, L., Dinarello, C.A., Towbin, H. & Andersson, U. (1992). Lipopolysaccharide induces human interleukin-1 receptor antagonist and interleukin-1 production in the same cell. *Eur J Immunol*, **22,** 2617-23.

Arai, K.I., Lee, F., Miyajima, A., Miyatake, S., Arai, N. & Yokota, T. (1990). Cytokines: coordinators of immune and inflammatory responses. *Annu Rev Biochem*, **59,** 783-836.

Arend, W.P., Malyak, M., Smith, M.F., Jr., Whisenand, T.D., Slack, J.L., Sims, J.E., Giri, J.G. & Dower, S.K. (1994). Binding of IL-1 alpha, IL-1 beta, and IL-1 receptor antagonist by soluble IL-1 receptors and levels of soluble IL-1 receptors in synovial fluids. *J Immunol*, **153,** 4766-74.

Arend, W.P., Welgus, H.G., Thompson, R.C. & Eisenberg, S.P. (1990). Biological properties of recombinant human monocyte-derived interleukin 1 receptor antagonist. *J Clin Invest*, **85,** 1694-7.

ArthritisResearchCampaign (2004). www.arc.org.uk.

Askwith, C.C., Benson, C.J., Welsh, M.J. & Snyder, P.M. (2001). DEG/ENaC ion channels involved in sensory transduction are modulated by cold temperature. *Proc Natl Acad Sci U S A*, **98,** 6459-63.

Averill, S., McMahon, S.B., Clary, D.O., Reichardt, L.F. & Priestley, J.V. (1995). Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur J Neurosci*, **7,** 1484-94.

Baamonde, A., Lastra, A., Villazon, M., Bordallo, J., Hidalgo, A. & Menendez, L. (2004). Involvement of endogenous endothelins in thermal and mechanical inflammatory hyperalgesia in mice. *Naunyn Schmiedebergs Arch Pharmacol*, **369,** 245-51.

Ballou, L.R., Botting, R.M., Goorha, S., Zhang, J. & Vane, J.R. (2000). Nociception in cyclooxygenase isozyme-deficient mice. *Proc Natl Acad Sci U S A*, **97,** 10272-6.

Banik, R.K., Kozaki, Y., Sato, J., Gera, L. & Mizumura, K. (2001). B2 receptor-mediated enhanced bradykinin sensitivity of rat cutaneous C-fiber nociceptors during persistent inflammation. *J Neurophysiol*, **86,** 2727-35.

Barland, P., Novikoff, A.B. & Hamerman, D. (1962). Electron microscopy of the human synovial membrane. *J Cell Biol*, **14,** 207-20.

Barnes, P.J. (2006). How corticosteroids control inflammation: Quintiles Prize Lecture 2005. *Br J Pharmacol*, **148,** 245-54.

Barrera, P., Boerbooms, A.M., van de Putte, L.B. & van der Meer, J.W. (1996). Effects of antirheumatic agents on cytokines. *Semin Arthritis Rheum*, **25,** 234-53.

Barrera, P., Joosten, L.A., den Broeder, A.A., van de Putte, L.B., van Riel, P.L. & van den Berg, W.B. (2001). Effects of treatment with a fully human anti-tumour necrosis factor alpha

monoclonal antibody on the local and systemic homeostasis of interleukin 1 and TNFalpha in patients with rheumatoid arthritis. *Ann Rheum Dis*, **60,** 660-9.

Bathon, J.M. (2005). Rheumatoid Arthritis- Pathophysiology: John Hopkins.

Baumann, H., Jahreis, G.P., Sauder, D.N. & Koj, A. (1984). Human keratinocytes and monocytes release factors which regulate the synthesis of major acute phase plasma proteins in hepatic cells from man, rat, and mouse. *J Biol Chem*, **259,** 7331-42.

Beaulieu, P. & Rice, A.S.C. (2002). Applied Physiology of Nociception. In *Acute Pain*. eds Rowbotham, D.J., Macintyre, P.E., Breivik, H., Campbell, W. & Eccleston, C. pp. 3-15. London: Arnold.

Beck, P.W. & Handwerker, H.O. (1974). Bradykinin and serotonin effects on various types of cutaneous nerve fibers. *Pflugers Arch*, **347,** 209-22.

Bell, J.K., McQueen, D.S. & Rees, J.L. (2004). Involvement of histamine H4 and H1 receptors in scratching induced by histamine receptor agonists in Balb C mice. *Br J Pharmacol*, **142,** 374-80.

Bellamy, N. (1989). Pain assessment in osteoarthritis: experience with the WOMAC osteoarthritis index. *Semin Arthritis Rheum*, **18,** 14-7.

Bellamy, N., Buchanan, W.W., Goldsmith, C.H., Campbell, J. & Stitt, L.W. (1988). Validation study of WOMAC: a health status instrument for measuring clinically important patient relevant outcomes to antirheumatic drug therapy in patients with osteoarthritis of the hip or knee. *J Rheumatol*, **15,** 1833-40.

Belmonte, C., Gallar, J., Pozo, M.A. & Rebollo, I. (1991). Excitation by irritant chemical substances of sensory afferent units in the cat's cornea. *J Physiol*, **437,** 709-25.

Belmonte, C. & Giraldez, F. (1981). Responses of cat corneal sensory receptors to mechanical and thermal stimulation. *J Physiol*, **321,** 355-68.

Bendele, A.M., Chlipala, E.S., Scherrer, J., Frazier, J., Sennello, G., Rich, W.J. & Edwards, C.K., 3rd (2000). Combination benefit of treatment with the cytokine inhibitors interleukin-1 receptor antagonist and PEGylated soluble tumor necrosis factor receptor type I in animal models of rheumatoid arthritis. *Arthritis Rheum*, **43,** 2648-59.

Bennett, D.L., Koltzenburg, M., Priestley, J.V., Shelton, D.L. & McMahon, S.B. (1998a). Endogenous nerve growth factor regulates the sensitivity of nociceptors in the adult rat. *Eur J Neurosci*, **10,** 1282-91.

Bennett, D.L., Michael, G.J., Ramachandran, N., Munson, J.B., Averill, S., Yan, Q., McMahon, S.B. & Priestley, J.V. (1998b). A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J Neurosci*, **18,** 3059-72.

Berenbaum, F., Jacques, C., Thomas, G., Corvol, M.T., Bereziat, G. & Masliah, J. (1996). Synergistic effect of interleukin-1 beta and tumor necrosis factor alpha on PGE2 production by articular chondrocytes does not involve PLA2 stimulation. *Exp Cell Res*, **222,** 379-84.

Bernardini, N., Sauer, S.K., Haberberger, R., Fischer, M.J. & Reeh, P.W. (2001). Excitatory nicotinic and desensitizing muscarinic (M2) effects on C-nociceptors in isolated rat skin. *J Neurosci*, **21,** 3295-302.

Berner, B., Akca, D., Jung, T., Muller, G.A. & Reuss-Borst, M.A. (2000). Analysis of Th1 and Th2 cytokines expressing CD4+ and CD8+ T cells in rheumatoid arthritis by flow cytometry. *J Rheumatol*, **27,** 1128-35.

Bernheim, H.A., Gilbert, T.M. & Stitt, J.T. (1980). Prostaglandin E levels in third ventricular cerebrospinal fluid of rabbits during fever and changes in body temperature. *J Physiol*, **301,** 69-78.

Bertolini, D.R., Votta, B., Hoffman, S. & Strassmann, G. (1994). Interleukin 6 production in fetal rat long bone cultures is correlated with PGE2 release and does not correlate with the extent of bone resorption. *Cytokine*, **6,** 368-75.

Besson, J.M. & Chaouch, A. (1987). Peripheral and spinal mechanisms of nociception. *Physiol Rev*, **67**, 67-186.

Bessou, P. & Perl, E.R. (1969). Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. *Journal Of Neurophysiology*, **32,** 1025-43.

Bevan, S. (1996a). Intracellular messengers and signal transduction in nociceptors. In *Neurobiology of nociceptors*. eds Belmonte, C. & Cervero, F. pp. 298-324. new York: Oxford University Press.

Bevan, S. (1996b). Signal transduction in nociceptive afferent neurons in inflammatory conditions. *Prog Brain Res*, **113,** 201-13.

Bevan, S. (1999). *Textbook of Pain*. New York: Churchill-Livingstone.

Bianchi, M., Maggi, R., Pimpinelli, F., Rubino, T., Parolaro, D., Poli, V., Ciliberto, G., Panerai, A.E. & Sacerdote, P. (1999). Presence of a reduced opioid response in interleukin-6 knock out mice. *Eur J Neurosci*, **11,** 1501-7.

Biedert, R.M., Stauffer, E. & Friederich, N.F. (1992). Occurrence of free nerve endings in the soft tissue of the knee joint. A histologic investigation. *Am J Sports Med*, **20,** 430-3.

Bileviciute, I., Lundeberg, T., Ekblom, A. & Theodorsson, E. (1993). Bilateral changes of substance P-, neurokinin A-, calcitonin gene-related peptide- and neuropeptide Y-like immunoreactivity in rat knee joint synovial fluid during acute monoarthritis. *Neurosci Lett*, **153,** 37-40.

Billingham, M.E. (1987). Cytokines as inflammatory mediators. *Br Med Bull*, **43,** 350-70.

Birrell, G.J., McQueen, D.S., Iggo, A., Coleman, R.A. & Grubb, B.D. (1991). PGI2-induced activation and sensitization of articular mechanonociceptors. *Neurosci Lett*, **124,** 5-8.

Birrell, G.J., McQueen, D.S., Iggo, A. & Grubb, B.D. (1990). The effects of 5-HT on articular sensory receptors in normal and arthritic rats. *Br J Pharmacol*, **101,** 715-21.

Bland-Ward, P.A. & Humphrey, P.P. (1997). Acute nociception mediated by hindpaw P2X receptor activation in the rat. *Br J Pharmacol*, **122,** 365-71.

Bleehen, T. & Keele, C.A. (1977). Observations on the algogenic actions of adenosine compounds on the human blister base preparation. *Pain*, **3,** 367-77.

Bolhuis, R.L., Schuit, H.R., Nooyen, A.M. & Ronteltap, C.P. (1978). Characterization of natural killer (NK) cells and killer (K) cells in human blood: discrimination between NK and K cell activities. *Eur J Immunol*, **8,** 731-40.

Bolon, B., Campagnuolo, G., Zhu, L., Duryea, D., Zack, D. & Feige, U. (2004). Interleukin-1beta and tumor necrosis factor-alpha produce distinct, time-dependent patterns of acute arthritis in the rat knee. *Vet Pathol*, **41,** 235-43.

Borden, P., Solymar, D., Sucharczuk, A., Lindman, B., Cannon, P. & Heller, R.A. (1996). Cytokine control of interstitial collagenase and collagenase-3 gene expression in human chondrocytes. *J Biol Chem*, **271,** 23577-81.

Bourde, O., Kiefer, R., Toyka, K.V. & Hartung, H.P. (1996). Quantification of interleukin-6 mRNA in wallerian degeneration by competitive reverse transcription polymerase chain reaction. *J Neuroimmunol*, **69,** 135-40.

Boyum, A. (1968). Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand J Clin Lab Invest Suppl*, **97,** 77-89.

Braun, H.A., Bade, H. & Hensel, H. (1980). Static and dynamic discharge patterns of bursting cold fibers related to hypothetical receptor mechanisms. *Pflugers Arch*, **386,** 1-9.

Brenn, D., Richter, F. & Schaible, H-G. (2007). Sensitization of unmyelinated sensory fibers of the joint nerve to mechanical stimuli by interleukin-6 in the rat. *Arth & Rheum.* **56,** 351-359.

Brennan, F.M., Chantry, D., Jackson, A., Maini, R. & Feldmann, M. (1989). Inhibitory effect of TNF alpha antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. *Lancet*, **2,** 244-7.

Brennan, F.M., Maini, R.N. & Feldmann, M. (1992). TNF alpha--a pivotal role in rheumatoid arthritis? *Br J Rheumatol*, **31,** 293-8.

Bresnihan, B. (2001). The safety and efficacy of interleukin-1 receptor antagonist in the treatment of rheumatoid arthritis. *Semin Arthritis Rheum*, **30,** 17-20.

Bresnihan, B., Alvaro-Gracia, J.M., Cobby, M., Doherty, M., Domljan, Z., Emery, P., Nuki, G., Pavelka, K., Rau, R., Rozman, B., Watt, I., Williams, B., Aitchison, R., McCabe, D. & Musikic, P. (1998). Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. *Arthritis Rheum*, **41,** 2196-204.

Bresnihan, B. & Cunnane, G. (1998). Interleukin-1 receptor antagonist. *Rheum Dis Clin North Am*, **24,** 615-28.

Bromley, M., Fisher, W.D. & Woolley, D.E. (1984). Mast cells at sites of cartilage erosion in the rheumatoid joint. *Ann Rheum Dis*, **43,** 76-9.

Burd, P.R., Thompson, W.C., Max, E.E. & Mills, F.C. (1995). Activated mast cells produce interleukin 13. *J Exp Med*, **181,** 1373-80.

Burgess, P.R. & Perl, E.R. (1967). Myelinated afferent fibres responding specifically to noxious stimulation of the skin. *Journal Of Physiology*, **190,** 541-62.

Burmester, G.R., Stuhlmuller, B., Keyszer, G. & Kinne, R.W. (1997). Mononuclear phagocytes and rheumatoid synovitis. Mastermind or workhorse in arthritis? *Arthritis Rheum*, **40,** 5-18.

Burnstock, G. (2000). P2X receptors in sensory neurons. *Br J Anaes,* **84,** 476-88.

Bush, K.A., Walker, J.S., Lee, C.S. & Kirkham, B.W. (2001). Cytokine expression and synovial pathology in the initiation and spontaneous resolution phases of adjuvant arthritis: interleukin-17 expression is upregulated in early disease. *Clin Exp Immunol*, **123,** 487-95.

Butler, D.M., Maini, R.N., Feldmann, M. & Brennan, F.M. (1995). Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist. *Eur Cytokine Netw*, **6,** 225-30.

Butler, S.H., Godefroy, F., Besson, J.M. & Weil-Fugazza, J. (1992). A limited arthritic model for chronic pain studies in the rat. *Pain*, **48,** 73-81.

Caldwell, J.R., Rapoport, R.J., Davis, J.C., Offenberg, H.L., Marker, H.W., Roth, S.H., Yuan, W., Eliot, L., Babul, N. & Lynch, P.M. (2002). Efficacy and safety of a once-daily morphine formulation in chronic, moderate-to-severe osteoarthritis pain: results from a randomized, placebo-controlled, double-blind trial and an open-label extension trial. *J Pain Symptom Manage*, **23,** 278-91.

Campagnuolo, G., Bolon, B., Zhu, L.I., Duryea, D., Feige, U. & Zack, D. (2003). IL-1{beta} and TNF-{alpha} Produce Divergent Acute Inflammatory and Skeletal Lesions in the Knees of Lewis Rats. *Ann NY Acad Sci*, **987,** 295-298.

Campbell, I.K., Roberts, L.J. & Wicks, I.P. (2003). Molecular targets in immune-mediated diseases: the case of tumour necrosis factor and rheumatoid arthritis. *Immunol Cell Biol*, **81,** 354-66.

Carlton, S.M. (2001). Peripheral excitatory amino acids. *Curr Opin Pharmacol*, **1,** 52-6.

Carlton, S.M. & Coggeshall, R.E. (2001). Peripheral capsaicin receptors increase in the inflamed rat hindpaw: a possible mechanism for peripheral sensitization. *Neurosci Lett*, **310,** 53-6.

Carlton, S.M., Hargett, G.L. & Coggeshall, R.E. (1995). Localization and activation of glutamate receptors in unmyelinated axons of rat glabrous skin. *Neurosci Lett*, **197,** 25-8.

Carlton, S.M., Zhou, S. & Coggeshall, R.E. (1998). Evidence for the interaction of glutamate and NK1 receptors in the periphery. *Brain Res*, **790,** 160-9.

Case, J.P., Lorberboum-Galski, H., Lafyatis, R., FitzGerald, D., Wilder, R.L. & Pastan, I. (1989). Chimeric cytotoxin IL2-PE40 delays and mitigates adjuvant-induced arthritis in rats. *Proc Natl Acad Sci U S A*, **86,** 287-91.

Castell, J.V., Gomez-Lechon, M.J., David, M., Hirano, T., Kishimoto, T. & Heinrich, P.C. (1988). Recombinant human interleukin-6 (IL-6/BSF-2/HSF) regulates the synthesis of acute phase proteins in human hepatocytes. *FEBS Lett*, **232,** 347-50.

Caswell, J.L., Middleton, D.M. & Gordon, J.R. (1999). Production and functional characterization of recombinant bovine interleukin-8 as a specific neutrophil activator and chemoattractant. *Vet Immunol Immunopathol*, **67,** 327-40.

Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., Koltzenburg, M., Basbaum, A.I. & Julius, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science*, **288,** 306-13.

Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J. & Julius, D.N.-U.C.F.C. (1999). A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature*, **398,** 436-441.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D. & Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature*, **389,** 816-24.

Cayphas, S., Van Damme, J., Vink, A., Simpson, R.J., Billiau, A. & Van Snick, J. (1987). Identification of an interleukin HP1-like plasmacytoma growth factor produced by L cells in response to viral infection. *J Immunol*, **139,** 2965-9.

Cervero, F. (1994). Sensory innervation of the viscera: peripheral basis of visceral pain. *Physiol Rev*, **74,** 95-138.

Cesare, P. & McNaughton, P. (1996). A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin. *Proc Natl Acad Sci U S A*, **93,** 15435-9.

Chandrasekhar, S., Harvey, A.K., Hrubey, P.S. & Bendele, A.M. (1990). Arthritis induced by interleukin-1 is dependent on the site and frequency of intraarticular injection. *Clin Immunol Immunopathol*, **55,** 382-400.

Chang, H. & Israel, H. (2005). Analysis of inflammatory mediators in temporomandibular joint synovial fluid lavage samples of symptomatic patients and asymptomatic controls. *J Oral Maxillofac Surg*, **63,** 761-5.

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M. & Yaksh, T.L. (1994). Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods*, **53,** 55-63.

Charles, P., Elliott, M.J., Davis, D., Potter, A., Kalden, J.R., Antoni, C., Breedveld, F.C., Smolen, J.S., Eberl, G., deWoody, K., Feldmann, M. & Maini, R.N. (1999). Regulation of cytokines, cytokine inhibitors, and acute-phase proteins following anti-TNF-alpha therapy in rheumatoid arthritis. *J Immunol*, **163,** 1521-8.

Chatham, W.W., Swaim, R., Frohsin, H., Jr., Heck, L.W., Miller, E.J. & Blackburn, W.D., Jr. (1993). Degradation of human articular cartilage by neutrophils in synovial fluid. *Arthritis Rheum*, **36,** 51-8.

Chedid, M. & Mizel, S.B. (1990). Involvement of cyclic AMP-dependent protein kinases in the signal transduction pathway for interleukin-1. *Mol Cell Biol*, **10,** 3824-7.

Chen, C.C., Akopian, A.N., Sivilotti, L., Colquhoun, D., Burnstock, G. & Wood, J.N. (1995). A P2X purinoceptor expressed by a subset of sensory neurons. *Nature*, **377,** 428-31.

Chen, E., Keystone, E.C. & Fish, E.N. (1993). Restricted cytokine expression in rheumatoid arthritis. *Arthritis Rheum*, **36,** 901-10.

Chikanza, I.C., Kingsley, G. & Panayi, G.S. (1995a). Peripheral blood and synovial fluid monocyte expression of interleukin 1 alpha and 1 beta during active rheumatoid arthritis. *J Rheumatol*, **22,** 600-6.

Chikanza, I.C., Roux-Lombard, P., Dayer, J.M. & Panayi, G.S. (1995b). Dysregulation of the in vivo production of interleukin-1 receptor antagonist in patients with rheumatoid arthritis. Pathogenetic implications. *Arthritis Rheum*, **38,** 642-8.

Cho, H., Shin, J., Shin, C.Y., Lee, S.Y. & Oh, U. (2002). Mechanosensitive ion channels in cultured sensory neurons of neonatal rats. *J Neurosci*, **22,** 1238-47.

Chomarat, P., Vannier, E., Dechanet, J., Rissoan, M.C., Banchereau, J., Dinarello, C.A. & Miossec, P. (1995). Balance of IL-1 receptor antagonist/IL-1 beta in rheumatoid synovium and its regulation by IL-4 and IL-10. *J Immunol*, **154,** 1432-9.

Choy, E.H., Isenberg, D.A., Garrood, T., Farrow, S., Ioannou, Y., Bird, H., Cheung, N., Williams, B., Hazleman, B., Price, R., Yoshizaki, K., Nishimoto, N., Kishimoto, T. & Panayi, G.S. (2002). Therapeutic benefit of blocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. *Arthritis Rheum*, **46,** 3143-50.

Choy, E.H. & Panayi, G.S. (2001). Cytokine pathways and joint inflammation in rheumatoid arthritis. *N Engl J Med*, **344,** 907-16.

Christodoulou, C. & Choy, E.H. (2006). Joint inflammation and cytokine inhibition in rheumatoid arthritis. *Clin Exp Med*, **6,** 13-9.

Chu, C.Q., Field, M., Allard, S., Abney, E., Feldmann, M. & Maini, R.N. (1992). Detection of cytokines at the cartilage/pannus junction in patients with rheumatoid arthritis: implications for the role of cytokines in cartilage destruction and repair. *Br J Rheumatol*, **31,** 653-61.

Cicala, C., Ianaro, A., Fiorucci, S., Calignano, A., Bucci, M., Gerli, R., Santucci, L., Wallace, J.L. & Cirino, G. (2000). NO-naproxen modulates inflammation, nociception and downregulates T cell response in rat Freund's adjuvant arthritis. *Br J Pharmacol*, **130,** 1399-405.

Clarke, D., Hughes, J. & Gasser, H.S. (1935). Afferent function in the group of nerve fibres of slowest conduction velocity. *American Journal of Physiology*, **114**.

Clarke, K.A., Heitmeyer, S.A., Smith, A.G. & Taiwo, Y.O. (1997). Gait analysis in a rat model of osteoarthrosis. *Physiol Behav*, **62,** 951-4.

Clawson, D.K., Souter, W.A., Carthum, C.J. & Hymen, M.L. (1971). Functional assessment of the rheumatoid hand. *Clin Orthop Relat Res*, **77,** 203-10.

Clayton, N.M., Oakley, I., Thompson, S., Wheeldon, A., Sargent, B. & Bountra, C. (1997). Validation of the dual channel weight averager as an instrument for the measurement of clinically relevant pain. *British Journal of Pharmacology*, **120**.

Cockayne, D.A., Hamilton, S.G., Zhu, Q.M., Dunn, P.M., Zhong, Y., Novakovic, S., Malmberg, A.B., Cain, G., Berson, A., Kassotakis, L., Hedley, L., Lachnit, W.G., Burnstock, G., McMahon, S.B. & Ford, A.P. (2000). Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature*, **407,** 1011-5.

Coderre, T.J. & Wall, P.D. (1988). Ankle joint urate arthritis in rats provides a useful tool for the evaluation of analgesic and anti-arthritic agents. *Pharmacol Biochem Behav*, **29,** 461-6.

Coggeshall, R.E., Hong, K.A., Langford, L.A., Schaible, H.G. & Schmidt, R.F. (1983). Discharge characteristics of fine medial articular afferents at rest and during passive movements of inflamed knee joints. *Brain Res*, **272,** 185-8.

Cohen, S.B., Katsikis, P.D., Chu, C.Q., Thomssen, H., Webb, L.M., Maini, R.N., Londei, M. & Feldmann, M. (1995). High level of interleukin-10 production by the activated T cell population within the rheumatoid synovial membrane. *Arthritis Rheum*, **38,** 946-52.

Cohen, S.B., Moreland, L.W., Cush, J.J., Greenwald, M.W., Block, S., Shergy, W.J., Hanrahan, P.S., Kraishi, M.M., Patel, A., Sun, G. & Bear, M.B. (2004). A multicentre, double blind, randomised, placebo controlled trial of anakinra (Kineret), a recombinant interleukin 1 receptor antagonist, in patients with rheumatoid arthritis treated with background methotrexate. *Ann Rheum Dis*, **63,** 1062-8.

Colbert, H.A., Smith, T.L., Bargmann, C.I. (1997). OSM-9, a novel protein with structural similarity to channels, is required for olfaction, mechanosensation, and olfactory adaptation in Caenorhabditis elegans. *J Neurosci,* **17,** 8259-69.

Collins, T. (1999). Acute and Chronic Inflammation. In *Pathologic Basis of Disease.* eds Cotran, R.S., Kumar, V. & Collins, T. London: W.B. Saunders Company.

Colotta, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., Giri, J.G., Dower, S.K., Sims, J.E. & Mantovani, A. (1993). Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science*, **261,** 472-5.

Colotta, F., Saccani, S., Giri, J.G., Dower, S.K., Sims, J.E., Introna, M. & Mantovani, A. (1996). Regulated expression and release of the IL-1 decoy receptor in human mononuclear phagocytes. *J Immunol*, **156,** 2534-41.

Conway, J.G., Andrews, R.C., Beaudet, B., Bickett, D.M., Boncek, V., Brodie, T.A., Clark, R.L., Crumrine, R.C., Leenitzer, M.A., McDougald, D.L., Han, B., Hedeen, K., Lin, P., Milla, M., Moss, M., Pink, H., Rabinowitz, M.H., Tippin, T., Scates, P.W., Selph, J., Stimpson, S.A., Warner, J. & Becherer, J.D. (2001). Inhibition of tumor necrosis factor-alpha (TNF-alpha) production and arthritis in the rat by GW3333, a dual inhibitor of TNF-alpha-converting enzyme and matrix metalloproteinases. *J Pharmacol Exp Ther*, **298,** 900-8.

Cook, C.D. & Moore, K.I. (2006). Effects of sex, hindpaw injection site and stimulus modality on nociceptive sensitivity in arthritic rats. *Physiol Behav*, **87,** 552-62.

Cooke, T.D., Hurd, E.R., Jasin, H.E., Bienenstock, J. & Ziff, M. (1975). Identification of immunoglobulins and complement in rheumatoid articular collagenous tissues. *Arthritis Rheum*, **18,** 541-51.

Corbel, C. & Melchers, F. (1984). The synergism of accessory cells and of soluble alpha-factors derived from them in the activation of B cells to proliferation. *Immunol Rev*, **78,** 51-74.

Corey, D.P., Garcia-Anoveros, J., Holt, J.R., Kwan, K.Y., Lin, S.Y., Vollrath, M.A., Amalfitano, A., Cheung, E.L., Derfler, B.H., Duggan, A., Geleoc, G.S., Gray, P.A., Hoffman, M.P., Rehm, H.L., Tamasauskas, D. & Zhang, D.S. (2004). TRPA1 is a candidate for the mechanosensitive transduction channel of vertebrate hair cells. *Nature*, **432,** 723-30.

Courtenay, J.S., Dallman, M.J., Dayan, A.D., Martin, A. & Mosedale, B. (1980). Immunisation against heterologous type II collagen induces arthritis in mice. *Nature*, **283,** 666-8.

Coutaux, A., Adam, F., Willer, J.C. & Le Bars, D. (2005). Hyperalgesia and allodynia: peripheral mechanisms. *Joint Bone Spine*, **72,** 359-71.

Craig, A.D., Heppelmann, B. & Schaible, H.G. (1988). The projection of the medial and posterior articular nerves of the cat's knee to the spinal cord. *J Comp Neurol*, **276,** 279-88.

Crofford, L.J., Wilder, R.L., Ristimaki, A.P., Sano, H., Remmers, E.F., Epps, H.R. & Hla, T. (1994). Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1 beta, phorbol ester, and corticosteroids. *J Clin Invest*, **93,** 1095-101.

Cui, J.G., Holmin, S., Mathiesen, T., Meyerson, B.A. & Linderoth, B. (2000). Possible role of inflammatory mediators in tactile hypersensitivity in rat models of mononeuropathy. *Pain*, **88,** 239-48.

Cui, J.G., Meyerson, B.A. & Linderoth, B. (1999). Opposite effects of spinal cord stimulation in different phases of carrageenan-induced hyperalgesia. *Eur J Pain*, **3,** 365-374.

Cunha, F.Q. & Ferreira, S.H. (2003). Peripheral hyperalgesic cytokines. *Adv Exp Med Biol*, **521,** 22-39.

Cunha, F.Q., Poole, S., Lorenzetti, B.B. & Ferreira, S.H. (1992). The pivotal role of tumour necrosis factor alpha in the development of inflammatory hyperalgesia. *Br J Pharmacol*, **107,** 660-4.

Cush, J.J., Splawski, J.B., Thomas, R., McFarlin, J.E., Schulze-Koops, H., Davis, L.S., Fujita, K. & Lipsky, P.E. (1995). Elevated interleukin-10 levels in patients with rheumatoid arthritis. *Arthritis Rheum*, **38,** 96-104.

Czlonkowski, A., Stein, C. & Herz, A. (1993). Peripheral mechanisms of opioid antinociception in inflammation: involvement of cytokines. *Eur J Pharmacol*, **242,** 229-35.

Danis, V.A., Franic, G.M., Rathjen, D.A. & Brooks, P.M. (1992). Circulating cytokine levels in patiets with rheumatoid arthritis: results of a double blind trial with sulphasalazine. *Ann of Rheum Dis*, **51**, 946-950.

Darian-Smith, I. & Johnson, K.O. (1977). Temperature sense in the primate. *Br Med Bull*, **33,** 143-8.

Dasgupta, B., Corkill, M., Kirkham, B., Gibson, T. & Panayi, G. (1992). Serial estimation of interleukin 6 as a measure of systemic disease in rheumatoid arthritis. *J Rheumatol*, **19,** 22-5.

Davidson, E.M., Coggeshall, R.E. & Carlton, S.M. (1997). Peripheral NMDA and non-NMDA glutamate receptors contribute to nociceptive behaviors in the rat formalin test. *Neuroreport*, **8,** 941-6.

Davis, J.B., Gray, J., Gunthorpe, M.J., Hatcher, J.P., Davey, P.T., Overend, P., Harries, M.H., Latcham, J., Clapham, C., Atkinson, K., Hughes, S.A., Rance, K., Grau, E., Harper, A.J., Pugh, P.L., Rogers, D.C., Bingham, S., Randall, A. & Sheardown, S.A. (2000). Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature*, **405,** 183-7.

Dayer, J.M. (2003). The pivotal role of interleukin-1 in the clinical manifestations of rheumatoid arthritis. *Rheumatology (Oxford)*, **42 Suppl 2,** ii3-10.

Dayer, J.M., Beutler, B. & Cerami, A. (1985). Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. *J Exp Med*, **162,** 2163-8.

Dayer, J.M., Breard, J., Chess, L. & Krane, S.M. (1979). Participation of monocyte-macrophages and lymphocytes in the production of a factor that stimulates collagenase and prostaglandin release by rheumatoid synovial cells. *J Clin Invest*, **64,** 1386-92.

Dayer, J.M., Krane, S.M., Russell, R.G. & Robinson, D.R. (1976). Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells. *Proc Natl Acad Sci U S A*, **73,** 945-9.

De Benedetti, F., Massa, M., Pignatti, P., Albani, S., Novick, D. & Martini, A. (1994). Serum soluble interleukin 6 (IL-6) receptor and IL-6/soluble IL-6 receptor complex in systemic juvenile rheumatoid arthritis. *J Clin Invest*, **93,** 2114-9.

deGroot, J., Zhou, S. & Carlton, S.M. (2000). Peripheral glutamate release in the hindpaw following low and high intensity sciatic stimulation. *Neuroreport,* **11,** 497-502.

de Hooge, A.S., van De Loo, F.A., Arntz, O.J. & van Den Berg, W.B. (2000). Involvement of IL-6, apart from its role in immunity, in mediating a chronic response during experimental arthritis. *Am J Pathol*, **157,** 2081-91.

De Jager, W., Hoppenreijs, P.A.H., Wulffraat, N.M., Wedderburn, L.R., Kuis, W. & Prakken, B.J. (2006). Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. *Ann Rheum Dis,* **66,** 589-598.

De Jongh, R.F., Vissers, K.C., Meert, T.F., Booij, L.H., De Deyne, C.S. & Heylen, R.J. (2003). The role of interleukin-6 in nociception and pain. *Anesth Analg*, **96,** 1096-103, table of contents.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G. & de Vries, J.E. (1991). Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med*, **174,** 1209-20.

DeLeo, J.A., Colburn, R.W., Nichols, M. & Malhotra, A. (1996). Interleukin-6-mediated hyperalgesia/allodynia and increased spinal IL-6 expression in a rat mononeuropathy model. *J Interferon Cytokine Res*, **16,** 695-700.

Deleuran, B.W. (1996). Cytokines in rheumatoid arthritis. Localization in arthritic joint tissue and regulation in vitro. *Scand J Rheumatol Suppl*, **104,** 1-34.

Deodhar, S.D., Dick, W.C., Hodgkinson, R. & Buchanan, W.W. (1973). Measurement of clinical response to anti-inflammatory drug therapy in rheumatoid arthritis. *Q J Med*, **42,** 387-401.

Dhaka, A., Viswanath, V. & Patapoutian, A. (2006). TRP Channels and Temperature Sensation. *Ann Rev Neurosci*, **29**, 135-61.

Dickenson, A.H. & Kieffer, B. (2006). Opiates: Basic Mechanisms, In Wall & Melzack's Textbook of Pain 5th Edition, McMahon, S.B. & Koltzenburg, M., Elsevier, Churchill Livingstone, p427-442

Di Giovine, F.S., Nuki, G. & Duff, G.W. (1988). Tumour necrosis factor in synovial exudates. *Ann Rheum Dis*, **47,** 768-72.

Dinarello, C.A. (2002). The IL-1 family and inflammatory diseases. *Clin Exp Rheumatol*, **20,** S1-13.

Dinarello, C.A. (1994a). Interleukin-1. *Adv Pharmacol*, **25,** 21-51.

Dinarello, C.A. (1994b). The interleukin-1 family: 10 years of discovery. *Faseb J*, **8,** 1314-25.

Dinarello, C.A. (1994c). Interleukin-1 in disease. *Keio J Med*, **43,** 131-6.

Dirajlal, S., Pauers, L.E. & Stucky, C.L. (2003). Differential response properties of IB(4)-positive and -negative unmyelinated sensory neurons to protons and capsaicin. *J Neurophysiol*, **89,** 513-24.

Djouhri, L., Koutsikou, S., Fang, X, McMullan, S. & Lawson, S.N. (2006). Spontaneous pain, both neuropathic and inflammatory, is related to frequency of spontaneous firing in intact c-fibre nociceptors, *Nerobiol of Dis,* **26,** 1281-1292.

Djouhri, L. & Lawson, S.N. (2004). Aβ-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. *Brain Res Rev*, **46**, 131-45.

Doak, G.J. & Sawynok, J. (1995). Complex role of peripheral adenosine in the genesis of the response to subcutaneous formalin in the rat. *Eur J Pharmacol*, **281,** 311-8.

Docherty, R.J., Yeats, J.C. & Piper, A.S. (1997). Capsazepine block of voltage-activated calcium channels in adult rat dorsal root ganglion neurones in culture. *Br J Pharmacol*, **121,** 1461-7.

Dolhain, R.J., Tak, P.P., Dijkmans, B.A., De Kuiper, P., Breedveld, F.C. & Miltenburg, A.M. (1998). Methotrexate reduces inflammatory cell numbers, expression of monokines and of adhesion molecules in synovial tissue of patients with rheumatoid arthritis. *Br J Rheumatol*, **37,** 502-8.

Donaldson, L.F. (1999). Unilateral arthritis: contralateral effects. *Trends in Neurosciences*, **22,** 495-496.

Donaldson, L.F., Seckl, J.R. & McQueen, D.S. (1993). A discrete adjuvant-induced monoarthritis in the rat: effects of adjuvant dose. *J Neurosci Methods*, **49,** 5-10.

Dowd, E., McQueen, D.S., Chessell, I.P. & Humphrey, P.P. (1998). P2X receptor-mediated excitation of nociceptive afferents in the normal and arthritic rat knee joint. *Br J Pharmacol*, **125,** 341-6.

Dray, A. (1997). Kinins and their receptors in hyperalgesia. *Can J Physiol Pharmacol*, **75,** 704-12.

Drelon, E., Gillet, P., Muller, N., Terlain, B. & Netter, P. (1994). Anti-inflammatory properties of IL-1 in carrageenan-induced paw oedema. *Agents Actions*, **41,** 50-2.

Drelon, E., Jouzeau, J.Y., Gillet, P., Gegout, P., Chevrier, D., Terlain, B. & Netter, P. (1992). Pro- and anti-inflammatory properties of human recombinant IL-1 beta during experimental arthritis in rats: 1. Dependence on dose and severity threshold. *Life Sci*, **51,** PL19-24.

Drevlow, B.E., Lovis, R., Haag, M.A., Sinacore, J.M., Jacobs, C., Blosche, C., Landay, A., Moreland, L.W. & Pope, R.M. (1996). Recombinant human interleukin-1 receptor type I in the treatment of patients with active rheumatoid arthritis. *Arthritis Rheum*, **39,** 257-65.

Drew, L.J., Wood, J.N. & Cesare, P. (2002). Distinct mechanosensitive properties of capsaicin-sensitive and -insensitive sensory neurons. *J Neurosci*, **22,** RC228.

Du, J., Koltzenburg, M. & Carlton, S.M. (2001). Glutamate-induced excitation and sensitization of nociceptors in rat glabrous skin. *Pain*, **89,** 187-98.

Eastgate, J.A., Symons, J.A., Wood, N.C., Capper, S.J. & Duff, G.W. (1991). Plasma levels of interleukin-1-alpha in rheumatoid arthritis. *Br J Rheumatol*, **30,** 295-7.

Eastgate, J.A., Symons, J.A., Wood, N.C., Grinlinton, F.M., di Giovine, F.S. & Duff, G.W. (1988). Correlation of plasma interleukin 1 levels with disease activity in rheumatoid arthritis. **2,** 706-9.

Ebinger, M., Schmidt, R.F. & Heppelmann, B. (2001). Composition of the medial and posterior articular nerves of the mouse knee joint. *Somatosens Mot Res*, **18,** 62-5.

Eccles, J.C. & Krnjevic, K. (1959). Potential changes recorded inside primary afferent fibres within the spinal cord. *J Physiol*, **149,** 250-73.

Edwards, C.K., 3rd (1999). PEGylated recombinant human soluble tumour necrosis factor receptor type I (r-Hu-sTNF-RI): novel high affinity TNF receptor designed for chronic inflammatory diseases. *Ann Rheum Dis*, **58 Suppl 1,** I73-81.

Egg, D. (1984). Concentrations of prostaglandins D2, E2, F2 alpha, 6-keto-F1 alpha and thromboxane B2 in synovial fluid from patients with inflammatory joint disorders and osteoarthritis. *Z Rheumatol*, **43,** 89-96.

Emery, P. (2001). Infliximab: a new treatment for rheumatoid arthritis. *Hosp Med*, **62,** 150-2.

England, S., Bevan, S. & Docherty, R.J. (1996). PGE2 modulates the tetrodotoxin-resistant sodium current in neonatal rat dorsal root ganglion neurones via the cyclic AMP-protein kinase A cascade. *J Physiol*, **495 (Pt 2),** 429-40.

Erlanger, J. & Gasser, H.S. (1937). *Electrical signs of nervous activity.* Philadelphia: University of Philadelphia Press.

Essner, R., Rhoades, K., McBride, W.H., Morton, D.L. & Economou, J.S. (1989). IL-4 down-regulates IL-1 and TNF gene expression in human monocytes. *J Immunol*, **142,** 3857-61.

Evans, C.H. & Robbins, P.D. (1994). The interleukin-1 receptor antagonist and its delivery by gene transfer. *Receptor*, **4,** 9-15.

Fattori, E., Cappelletti, M., Costa, P., Sellitto, C., Cantoni, L., Carelli, M., Faggioni, R., Fantuzzi, G., Ghezzi, P. & Poli, V. (1994). Defective inflammatory response in interleukin 6-deficient mice. *J Exp Med*, **180,** 1243-50.

Feige, U., Hu, Y.L., Gasser, J., Campagnuolo, G., Munyakazi, L. & Bolon, B. (2000). Anti-interleukin-1 and anti-tumor necrosis factor-alpha synergistically inhibit adjuvant arthritis in Lewis rats. *Cell Mol Life Sci*, **57,** 1457-70.

Feige, U., Karbowski, A., Rordorf-Adam, C. & Pataki, A. (1989). Arthritis induced by continuous infusion of hr-interleukin-1 alpha into the rabbit knee-joint. *Int J Tissue React*, **11,** 225-38.

Feldmann, M., Brennan, F.M., Chantry, D., Haworth, C., Turner, M., Abney, E., Buchan, G., Barrett, K., Barkley, D., Chu, A. & et al. (1990). Cytokine production in the rheumatoid joint: implications for treatment. *Ann Rheum Dis*, **49,** 480-6.

Feldmann, M., Brennan, F.M. & Maini, R.N. (1996). Rheumatoid arthritis. *Cell*, **85,** 307-10.

Feldmann, M. & Maini, R.N. (2003). Lasker Clinical Medical Research Award. TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases. *Nat Med*, **9,** 1245-50.

Ferreira, S.H. (1972). Prostaglandins, aspirin-like drugs and analgesia. *Nat New Biol*, **240,** 200-3.

Ferreira, S.H., Lorenzetti, B.B., Bristow, A.F. & Poole, S. (1988). Interleukin-1 beta as a potent hyperalgesic agent antagonized by a tripeptide analogue. *Nature*, **334,** 698-700.

Ferreira, S.H. & Vane, J.R. (1967). Prostaglandins: their disappearance from and release into the circulation. *Nature*, **216,** 868-73.

Field, K.J., White, W.J. & Lang, C.M. (1993). Anaesthetic effects of chloral hydrate, pentobarbitone and urethane in adult male rats. *Lab Anim*, **27,** 258-69.

Field, M., Chu, C., Feldmann, M. & Maini, R.N. (1991). Interleukin-6 localisation in the synovial membrane in rheumatoid arthritis. *Rheumatol Int*, **11,** 45-50.

Fields, H.L., Basbaum, A.I. & Heinricher, M.M. (2006). Central nervous system mechanisms of pain modulation. In *Textbook of Pain*. eds Wall, P.D. & Melzack, R. pp. 35-48. London: Churchill Livingstone.

Firestein, G.S. (2003). Evolving concepts of rheumatoid arthritis. *Nature*, **423,** 356-61.

Firestein, G.S., Alvaro-Gracia, J.M. & Maki, R. (1990). Quantitative analysis of cytokine gene expression in rheumatoid arthritis. *J Immunol*, **144,** 3347-53.

Firestein, G.S., Boyle, D.L., Yu, C., Paine, M.M., Whisenand, T.D., Zvaifler, N.J. & Arend, W.P. (1994). Synovial interleukin-1 receptor antagonist and interleukin-1 balance in rheumatoid arthritis. *Arthritis Rheum*, **37,** 644-52.

Firestein, G.S. & Zvaifler, N.J. (2002). How important are T cells in chronic rheumatoid synovitis?: II. T cell-independent mechanisms from beginning to end. *Arthritis Rheum*, **46,** 298-308.

Fischer, E., Van Zee, K.J., Marano, M.A., Rock, C.S., Kenney, J.S., Poutsiaka, D.D., Dinarello, C.A., Lowry, S.F. & Moldawer, L.L. (1992). Interleukin-1 receptor antagonist circulates in experimental inflammation and in human disease. *Blood*, **79,** 2196-200.

Fischer, H.G., Frosch, S., Reske, K. & Reske-Kunz, A.B. (1988). Granulocyte-macrophage colony-stimulating factor activates macrophages derived from bone marrow cultures to synthesis of MHC class II molecules and to augmented antigen presentation function. *J Immunol*, **141,** 3882-8.

Follenfant, R.L., Nakamura-Craig, M., Henderson, B. & Higgs, G.A. (1989). Inhibition by neuropeptides of interleukin-1 beta-induced, prostaglandin-independent hyperalgesia. *Br J Pharmacol*, **98,** 41-3.

Fong, K.Y., Boey, M.L., Koh, W.H. & Feng, P.H. (1994). Cytokine concentrations in the synovial fluid and plasma of rheumatoid arthritis patients: correlation with bony erosions. *Clin Exp Rheumatol*, **12,** 55-8.

Fox, A.J., Barnes, P.J., Urban, L. & Dray, A. (1993). An in vitro study of the properties of single vagal afferents innervating guinea-pig airways. *J Physiol*, **469,** 21-35.

Fox, D.A. (2000). Cytokine blockade as a new strategy to treat rheumatoid arthritis: inhibition of tumor necrosis factor. *Arch Intern Med*, **160,** 437-44.

Fox, D.A. (1997). The role of T cells in the immunopathogenesis of rheumatoid arthritis: new perspectives. *Arthritis Rheum*, **40,** 598-609.

Fredholm, B.B., AP, I.J., Jacobson, K.A., Klotz, K.N. & Linden, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev*, **53,** 527-52.

Freeman, M.A.R. & Wyke, B. (1967). The innervatio of the knee joint: an anatomical and historical study in the cat. *The journal of anatomy*, **101,** 505-532.

Frei, K., Malipiero, U.V., Leist, T.P., Zinkernagel, R.M., Schwab, M.E. & Fontana, A. (1989). On the cellular source and function of interleukin 6 produced in the central nervous system in viral diseases. *Eur J Immunol*, **19,** 689-94.

Freund, J. (1956). The mode of action of immunologic adjuvants. *Adv Tuberc Res*, **7.**

Freund, J. (1947). Some aspects of active immunization. *Annual Review of Microbiology*, **1,** 291-309.

Fries, J.F., Spitz, P., Kraines, R.G. & Holman, H.R. (1980). Measurement of patient outcome in arthritis. *Arthritis Rheum*, **23,** 137-45.

Fukuoka, H., Kawatani, M., Hisamitsu, T. & Takeshige, C. (1994). Cutaneous hyperalgesia induced by peripheral injection of interleukin-1 beta in the rat. *Brain Res*, **657,** 133-40.

Fulkerson, J.P. & Damiano, P. (1983). Effect of prostaglandin E2 on adult pig articular cartilage slices in culture. *Clin Orthop Relat Res*, 266-9.

Furst, D.E. (2004). Anakinra: review of recombinant human interleukin-I receptor antagonist in the treatment of rheumatoid arthritis. *Clin Ther*, **26,** 1960-75.

Furst, D.E., Schiff, M.H., Fleischmann, R.M., Strand, V., Birbara, C.A., Compagnone, D., Fischkoff, S.A. & Chartash, E.K. (2003). Adalimumab, a fully human anti tumor necrosis factor-alpha monoclonal antibody, and concomitant standard antirheumatic therapy for the treatment of rheumatoid arthritis: results of STAR (Safety Trial of Adalimumab in Rheumatoid Arthritis). *J Rheumatol*, **30,** 2563-71.

Gardner, E. (1944). The distribution and termination of nerves in the knee joint of the cat. *The journal of comparitive neurology*, **80,** 11-32.

Garell, P.C., McGillis, S.L. & Greenspan, J.D. (1996). Mechanical response properties of nociceptors innervating feline hairy skin. *J Neurophysiol*, **75,** 1177-89.

Garnsworthy, R.K., Gully, R.L., Kenins, P., Mayfield, R.J. & Westerman, R.A. (1988). Identification of the physical stimulus and the neural basis of fabric-evoked prickle. *J Neurophysiol*, **59,** 1083-97.

Garrison, L. & McDonnell, N.D. (1999). Etanercept: therapeutic use in patients with rheumatoid arthritis. *Ann Rheum Dis*, **58 Suppl 1,** I65-9.

Gasser, H.S. & Erlanger, J. (1927). The role played by the sizes of the constitunt fibres of a nerve trunk in determining the form of its action potential wave. *American Journal of Physiology*, **80,** 522-547.

Gauldie, S.D., McQueen, D.S., Clarke, C.J. & Chessell, I.P. (2004). A robust model of adjuvant-induced chronic unilateral arthritis in two mouse strains. *J Neurosci Methods*, **139,** 281-91.

Gauriau, C. & Bernard, J.F. (2004). Posterior triangular thalamic neurons convey nociceptive messages to the secondary somatosensory and insular cortices in the rat. *J Neurosci*, **24,** 752-61.

Gegout-Pottie, P., Philippe, L., Simonin, M.A., Guingamp, C., Gillet, P., Netter, P. & Terlain, B. (1999). Biotelemetry: an original approach to experimental models of inflammation. *Inflamm Res*, **48,** 417-24.

Gerlag, D.M., Haringman, J.J., Smeets, T.J., Zwinderman, A.H., Kraan, M.C., Laud, P.J., Morgan, S., Nash, A.F. & Tak, P.P. (2004). Effects of oral prednisolone on biomarkers in synovial tissue and clinical improvement in rheumatoid arthritis. *Arthritis Rheum*, **50,** 3783-91.

Ghadially, F.N. & Roy, S. (1966). Ultrastructure of rabbit synovial membrane. *Ann Rheum Dis*, **25,** 318-26.

Gibson, T. & Clark, B. (1985). Use of simple analgesics in rheumatoid arthritis. *ANN. RHEUM. DIS.*, **44,** 27-29.

Gillis, S., Ferm, M.M., Ou, W. & Smith, K.A. (1978). T cell growth factor: parameters of production and a quantitative microassay for activity. *J Immunol*, **120,** 2027-32.

Giraldez, F., Geijo, E. & Belmonte, C. (1979). Response characteristics of corneal sensory fibers to mechanical and thermal stimulation. *Brain Res*, **177,** 571-6.

Girard, D., Paquin, R., Naccache, P.H. & Beaulieu, A.D. (1996). Effects of interleukin-13 on human neutrophil functions. *J Leukoc Biol*, **59,** 412-9.

Glynn, L.E. (1968). The chronicity of inflammation and its significance in rheumatoid arthritis. *Ann Rheum Dis*, **27,** 105-21.

Gold, M.S., Reichling, D.B., Shuster, M.J. & Levine, J.D. (1996). Hyperalgesic agents increase a tetrodotoxin-resistant Na+ current in nociceptors. *Proc Natl Acad Sci U S A*, **93,** 1108-12.

Goldenberg, M.M. (1999). Etanercept, a novel drug for the treatment of patients with severe, active rheumatoid arthritis. *Clin Ther*, **21,** 75-87; discussion 1-2.

Goodman, M.B., Lumpkin, E.A., Ricci, A. Tracey, W.D., Kernan, M. & Nicholson, T. Molecules and mechanisms of mechanotransduction. *J Neurosci*, **24,** 9220-2.

Goodson, T., Morgan, S.L., Carlee, J.R. & Baggott, J.E. (2003). The energy cost of adjuvant-induced arthritis in rats. *Arthritis Rheum*, **48,** 2979-82.

Goronzy, J.J. & Weyand, C.M. (1995). T cells in rheumatoid arthritis. Paradigms and facts. *Rheum Dis Clin North Am*, **21,** 655-74.

Gottschalk, A. & Smith, D.S. (2001). New Concepts inAcute Pain Therapy: Preemptive Analgesia. *American Family Physician*, **63,** 1979-1984.

Gracely, R.H., Grant, M.A. & Giesecke, T. (2003). Evoked pain measures in fibromyalgia. *Best Pract Res Clin Rheumatol*, **17,** 593-609.

Gravallese, E.M. (2002). Bone destruction in arthritis. *Ann Rheum Dis*, **61 Suppl 2,** ii84-6.

Gravallese, E.M. & Goldring, S.R. (2000). Cellular mechanisms and the role of cytokines in bone erosions in rheumatoid arthritis. *Arthritis Rheum*, **43,** 2143-51.

Graves, B.J., Hatada, M.H., Hendrickson, W.A., Miller, J.K., Madison, V.S. & Satow, Y. (1990). Structure of interleukin 1 alpha at 2.7-A resolution. *Biochemistry*, **29,** 2679-84.

Gray, H. (1918). *Anatomy of the Human Body*. Philadelphia: Lea & Febiger.

Greenfeder, S.A., Nunes, P., Kwee, L., Labow, M., Chizzonite, R.A. & Ju, G. (1995). Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. *J Biol Chem*, **270,** 13757-65.

Greenwood, F., Horiuchi, H. & Matthews, B. (1972). Electrophysiological evidence on the types of nerve fibres excited by electrical stimulation of teeth with a pulp tester. *Arch Oral Biol*, **17,** 701-9.

Gronich, J., Konieczkowski, M., Gelb, M.H., Nemenoff, R.A. & Sedor, J.R. (1994). Interleukin 1 alpha causes rapid activation of cytosolic phospholipase A2 by phosphorylation in rat mesangial cells. *J Clin Invest*, **93,** 1224-33.

Groopman, J.E., Molina, J.M. & Scadden, D.T. (1989). Hematopoietic growth factors. Biology and clinical applications. *N Engl J Med*, **321,** 1449-59.

Grubb, B.D., Birrell, G.J., McQueen, D.S. & Iggo, A. (1991). The role of PGE2 in the sensitization of mechanoreceptors in normal and inflamed ankle joints of the rat. *Exp Brain Res*, **84,** 383-92.

Guay, J., Bateman, K., Gordon, R., Mancini, J. & Riendeau, D. (2004). Carrageenan-induced Paw Edema in Rat Elicits a Predominant Prostaglandin E2 (PGE2) Response in the Central Nervous System Associated with the Induction of Microsomal PGE2 Synthase-1. *J. Biol. Chem.*, **279,** 24866-24872.

Guerne, P.A., Carson, D.A. & Lotz, M. (1990). IL-6 production by human articular chondrocytes. Modulation of its synthesis by cytokines, growth factors, and hormones in vitro. *J Immunol*, **144,** 499-505.

Guerne, P.A., Zuraw, B.L., Vaughan, J.H., Carson, D.A. & Lotz, M. (1989). Synovium as a source of interleukin 6 in vitro. Contribution to local and systemic manifestations of arthritis. *J Clin Invest*, **83,** 585-92.

Guilbaud, G., Iggo, A. & Tegner, R. (1985). Sensory receptors in ankle joint capsules of normal and arthritic rats. *Exp Brain Res*, **58,** 29-40.

Guo, A., Simone, D.A., Stone, L.S., Fairbanks, C.A., Wang, J. & Elde, R. (2001). Developmental shift of vanilloid receptor 1 (VR1) terminals into deeper regions of the superficial dorsal horn: correlation with a shift from TrkA to Ret expression by dorsal root ganglion neurons. *Eur J Neurosci*, **14,** 293-304.

Guo, A., Vulchanova, L., Wang, J., Li, X. & Elde, R. (1999). Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. *Eur J Neurosci*, **11,** 946-58.

Gurr, E., Delbruck, A. & Drommer, W. (1988). Cartilage destruction mechanisms: initial changes in the proteoglycan pattern in Erysipelas arthritis of pigs in vivo and chondrocyte repair reaction in vitro. *Agents Actions*, **23,** 42-4.

Halata, Z., Rettig, T. & Schulze, W. (1985). The ultrastructure of sensory nerve endings in the human knee joint capsule. *Anat Embryol (Berl)*, **172,** 265-75.

Hamill, O.P., Lane, J.W. & McBride, D.W., Jr. (1992). Amiloride: a molecular probe for mechanosensitive channels. *Trends Pharmacol Sci*, **13,** 373-6.

Han, J.S., Bird, G.C., Li, W., Jones, J. & Neugebauer, V. (2005). Computerized analysis of audible and ultrasonic vocalizations of rats as a standardized measure of pain-related behavior. *J Neurosci Methods*, **141,** 261-9.

Handwerker, H.O. (2006). Nociceptors: neurogenic inflammation. In *Handbook of Clinical Neurology*. eds Cervero, F. & Jensen, T.S. London: Elsevier.

Handwerker, H.O. & Neher, K.D. (1976). Characteristics of C-fibre receptors in the cat's foot responding to stepwise increase of skin temperature ot noxious levels. *Pflugers Arch*, **365,** 221-9.

Haraoui, B., Pelletier, J.P., Cloutier, J.M., Faure, M.P. & Martel-Pelletier, J. (1991). Synovial membrane histology and immunopathology in rheumatoid arthritis and osteoarthritis. In vivo effects of antirheumatic drugs. *Arthritis Rheum*, **34,** 153-63.

Harigai, M., Hara, M., Kitani, A., Norioka, K., Hirose, T., Hirose, W., Suzuki, K., Kawakami, M., Masuda, K., Shinmei, M. & et al. (1991). Interleukin 1 and tumor necrosis factor-alpha synergistically increase the production of interleukin 6 in human synovial fibroblast. *J Clin Lab Immunol*, **34,** 107-13.

Haringman, J.J., Gerlag, D.M., Zwinderman, A.H., Smeets, T.J., Kraan, M.C., Baeten, D., McInnes, I.B., Bresnihan, B. & Tak, P.P. (2005). Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis. *Ann Rheum Dis*, **64,** 834-8.

Harris, E.D., Jr. (1990). Rheumatoid arthritis. Pathophysiology and implications for therapy. *N Engl J Med*, **322,** 1277-89.

Hase, C.C., Le Dain, A.C. & Martinac, B. (1995). Purification and functional reconstitution of the recombinant large mechanosensitive ion channel (MscL) of Escherichia coli. *J Biol Chem*, **270,** 18329-34.

Hata, H., Sakaguchi, N., Yoshitomi, H., Iwakura, Y., Sekikawa, K., Azuma, Y., Kanai, C., Moriizumi, E., Nomura, T., Nakamura, T. & Sakaguchi, S. (2004). Distinct contribution of IL-6, TNF-alpha, IL-1, and IL-10 to T cell-mediated spontaneous autoimmune arthritis in mice. *J Clin Invest*, **114,** 582-8.

Hawkey, C.J. (2001). COX-1 and COX-2 inhibitors. *Best Prac & Clin Gastroent,* **15,** 801-820.

Heiberg, T. & Kvien, T.K. (2002). Preferences for improved health examined in 1,024 patients with rheumatoid arthritis: pain has highest priority. *Arthritis Rheum*, **47,** 391-7.

Heidenreich, S., Gong, J.H., Schmidt, A., Nain, M. & Gemsa, D. (1989). Macrophage activation by granulocyte/macrophage colony-stimulating factor. Priming for enhanced release of tumor necrosis factor-alpha and prostaglandin E2. *J Immunol*, **143,** 1198-205.

Heinbecker, P., Bishop, G.H. & O'Leary, J. (1932). Fibres in mixed nerves and their dorsal roots responsible for pain. *Proceedings of the society for experimental biology and medicine*, **29**.

Helyes, Z., Pinter, E. & Szolcsanyi, J. (2003). Pharmacological targets for the inhibition of neurogenic inflammation. *Curr Med Chem AIAA*, **2,** 191-218.

Henderson, B. & Pettipher, E.R. (1989). Arthritogenic actions of recombinant IL-1 and tumour necrosis factor alpha in the rabbit: evidence for synergistic interactions between cytokines in vivo. *Clin Exp Immunol*, **75,** 306-10.

Hensel, H. & Zotterman, Y. (1951). The effect of menthol on the thermoreceptors. *Acta Physiol Scand*, **24,** 27-34.

Heppelmann, B. (1997). Anatomy and histology of joint innervation. *J Peripher Nerv Syst*, **2,** 5-16.

Heppelmann, B., Heuss, C. & Schmidt, R.F. (1988a). Fiber size distribution of myelinated and unmyelinated axons in the medial and posterior articular nerves of the cat's knee joint. *Somatosens Res*, **5,** 273-81.

Heppelmann, B., Messlinger, K. & Schmidt, R.F. (1988b). Morphological characteristics of the innervation of the cat's knee joint. *Agents Actions*, **25,** 225-7.

Heppelmann, B., Pfeffer, A., Schaible, H.G. & Schmidt, R.F. (1986). Effects of acetylsalicylic acid and indomethacin on single groups III and IV sensory units from acutely inflamed joints. *Pain*, **26,** 337-51.

Herbert, M.K. & Schmidt, R.F. (1992). Activation of normal and inflamed fine articular afferent units by serotonin. *Pain*, **50,** 79-88.

Hierl, T., Borcsok, I., Sommer, U., Ziegler, R. & Kasperk, C. (1998). Regulation of interleukin-6 expression in human osteoblastic cells in vitro. *Exp Clin Endocrinol Diabetes*, **106,** 324-33.

Hildebrand, C., Oqvist, G., Brax, L. & Tuisku, F. (1991). Anatomy of the rat knee joint and fibre composition of a major articular nerve. *Anat Rec*, **229,** 545-55.

Hilliges, M., Weidner, C., Schmelz, M., Schmidt, R., Orstavik, K., Torebjork, E. & Handwerker, H. (2002). ATP responses in human C nociceptors. *Pain*, **98,** 59-68.

Hirano, T. (1992). Interleukin-6 and its relation to inflammation and disease. *Clin Immunol Immunopathol*, **62,** S60-5.

Hirano, T., Matsuda, T., Turner, M., Miyasaka, N., Buchan, G., Tang, B., Sato, K., Shimizu, M., Maini, R., Feldmann, M. & et al. (1988). Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis. *Eur J Immunol*, **18,** 1797-801.

Hirano, T., Taga, T., Nakano, N., Yasukawa, K., Kashiwamura, S., Shimizu, K., Nakajima, K., Pyun, K.H. & Kishimoto, T. (1985). Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2). *Proc Natl Acad Sci U S A*, **82,** 5490-4.

Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., Koyama, K., Iwamatsu, A. & et al. (1986). Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature*, **324,** 73-6.

Hochberg, M.C., Tracy, J.K., Hawkins-Holt, M. & Flores, R.H. (2003). Comparison of the efficacy of the tumour necrosis factor alpha blocking agents adalimumab, etanercept, and infliximab when added to methotrexate in patients with active rheumatoid arthritis. *Ann Rheum Dis*, **62 Suppl 2,** ii13-6.

Holmgren, M., Hedner, J., Mellstrand, T., Nordberg, G. & Hedner, T. (1986). Characterization of the antinociceptive effects of some adenosine analogues in the rat. *Naunyn Schmiedebergs Arch Pharmacol*, **334,** 290-3.

Holzheimer, R.G. & Steinmetz, W. (2000). Local and systemic concentrations of pro- and anti-inflammatory cytokines in human wounds. *Eur J Med Res*, **5,** 347-55.

Hom, J.T., Bendele, A.M. & Carlson, D.G. (1988). In vivo administration with IL-1 accelerates the development of collagen-induced arthritis in mice. *J Immunol*, **141,** 834-41.

Hom, J.T., Cole, H. & Bendele, A.M. (1990). Interleukin 1 enhances the development of spontaneous arthritis in MRL/lpr mice. *Clin Immunol Immunopathol*, **55,** 109-19.

Hopkins, S.J., Humphreys, M. & Jayson, M.I. (1988). Cytokines in synovial fluid. I. The presence of biologically active and immunoreactive IL-1. *Clin Exp Immunol*, **72,** 422-7.

Hopkins, S.J. & Meager, A. (1988). Cytokines in synovial fluid: II. The presence of tumour necrosis factor and interferon. *Clin Exp Immunol*, **73,** 88-92.

Horai, R., Saijo, S., Tanioka, H., Nakae, S., Sudo, K., Okahara, A., Ikuse, T., Asano, M. Iwakura, Y. (2000). Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. *J Exp Med.* **191,** 313-20.

Houssiau, F.A. (1995). Cytokines in rheumatoid arthritis. *Clin Rheumatol*, **14 Suppl 2,** 10-3.

Houssiau, F.A., Devogelaer, J.P., Van Damme, J., de Deuxchaisnes, C.N. & Van Snick, J. (1988). Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides. *Arthritis Rheum*, **31,** 784-8.

Hughes, D.I., Scott, D.T., Todd, A.J. & Riddell, J.S. (2003). Lack of evidence for sprouting of Abeta afferents into the superficial laminas of the spinal cord dorsal horn after nerve section. *J Neurosci*, **23,** 9491-9.

Hultgren, O., Eugster, H.P., Sedgwick, J.D., Korner, H. & Tarkowski, A. (1998). TNF/lymphotoxin-alpha double-mutant mice resist septic arthritis but display increased mortality in response to Staphylococcus aureus. *J Immunol*, **161,** 5937-42.

Hunt, S.P. & Mantyh, P.W. (2001). The molecular dynamics of pain control. *Nat Rev Neurosci.* **2,** 83-91.

Hunt, S.M., McEwen, J. & McKenna, S.P. (1985). Measuring health status: a new tool for clinicians and epidemiologists. *J R Coll Gen Pract*, **35,** 185-8.

Huskisson, E.C. (1974). Measurement of pain. *Lancet*, **2,** 1127-31.

Hwang, S.W., Cho, H., Kwak, J., Lee, S.Y., Kang, C.J., Jung, J., Cho, S., Min, K.H., Suh, Y.G., Kim, D. & Oh, U. (2000). Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. *Proc Natl Acad Sci U S A*, **97,** 6155-60.

Ikejima, T., Okusawa, S., Ghezzi, P., van der Meer, J.W. & Dinarello, C.A. (1990). Interleukin-1 induces tumor necrosis factor (TNF) in human peripheral blood mononuclear cells in vitro and a circulating TNF-like activity in rabbits. *J Infect Dis*, **162,** 215-23.

Inoue, A., Ikoma, K., Morioka, N., Kumagai, K., Hashimoto, T., Hide, I. & Nakata, Y. (1999). Interleukin-1beta induces substance P release from primary afferent neurons through the cyclooxygenase-2 system. *J Neurochem*, **73,** 2206-13.

Inoue, M., Minami, M., Matsumoto, M., Kishimoto, T. & Akira, S. (1997). The amino acid residues immediately carboxyl-terminal to the tyrosine phosphorylation site contribute to interleukin 6-specific activation of signal transducer and activator of transcription 3. *J Biol Chem*, **272,** 9550-5.

Ishihara, K. & Hirano, T. (2002). IL-6 in autoimmune disease and chronic inflammatory proliferative disease. *Cytokine Growth Factor Rev*, **13,** 357-68.

Ishimi, Y., Miyaura, C., Jin, C.H., Akatsu, T., Abe, E., Nakamura, Y., Yamaguchi, A., Yoshiki, S., Matsuda, T., Hirano, T. & et al. (1990). IL-6 is produced by osteoblasts and induces bone resorption. *J Immunol*, **145,** 3297-303.

Isomaki, P., Luukkainen, R., Saario, R., Toivanen, P. & Punnonen, J. (1996). Interleukin-10 functions as an antiinflammatory cytokine in rheumatoid synovium. *Arthritis Rheum*, **39,** 386-95.

Isomaki, P. & Punnonen, J. (1997). Pro- and anti-inflammatory cytokines in rheumatoid arthritis. *Ann Med*, **29,** 499-507.

Ito, A., Itoh, Y., Sasaguri, Y., Morimatsu, M. & Mori, Y. (1992). Effects of interleukin-6 on the metabolism of connective tissue components in rheumatoid synovial fibroblasts. *Arthritis Rheum*, **35,** 1197-201.

Ito, Y., Yamamoto, M., Li, M., Doyu, M., Tanaka, F., Mutch, T., Mitsuma, T. & Sobue, G. (1998). Differential temporal expression of mRNAs for ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), and their receptors (CNTFR alpha, LIFR beta, IL-6R alpha and gp130) in injured peripheral nerves. *Brain Res*, **793,** 321-7.

Jacobs, C., Young, D., Tyler, S., Callis, G., Gillis, S. & Conlon, P.J. (1988). In vivo treatment with IL-1 reduces the severity and duration of antigen-induced arthritis in rats. *J Immunol*, **141,** 2967-74.

Jancso, N., Jancso-Gabor, A. & Szolcsanyi, J. (1967). Direct evidence for neurogenic inflammation
and its prevention by denervation and by pretreatment
with capsaicin. *British Journal of Pharmacology*, **38,** 138-51.

Jenkins, J.K., Malyak, M. & Arend, W.P. (1994). The effects of interleukin-10 on interleukin-1 receptor antagonist and interleukin-1 beta production in human monocytes and neutrophils. *Lymphokine Cytokine Res*, **13,** 47-54.

Ji, R.R., Kohno, T., Moore, K.A. & Woolf, C.J. (2003). Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends Neurosci*, **26,** 696-705.

Jobanputra, P., Barton, P., Bryan, S. & Burls, A. (2002). The effectiveness of infliximab and etanercept for the treatment of rheumatoid arthritis: a systematic review and economic evaluation. *Health Technol Assess*, **6,** 1-110.

Johnson, K.O., Darian-Smith, I., LaMotte, C., Johnson, B. & Oldfield, S. (1979). Coding of incremental changes in skin temperature by a population of warm fibers in the monkey: correlation with intensity discrimination in man. *J Neurophysiol*, **42,** 1332-53.

Jones, N.G., Slater, R., Cadiou, H., McNaughton, P. & McMahon, S.B. (2004). Acid-induced pain and its modulation in humans. *J Neurosci*, **24,** 10974-9.

Jones, S.A., Horiuchi, S., Topley, N., Yamamoto, N. & Fuller, G.M. (2001). The soluble interleukin 6 receptor: mechanisms of production and implications in disease. *Faseb J*, **15,** 43-58.

Joosten, L., Helsen, M.M., van de Loo, F.A. & van den Berg, W.B. (1996). Anticytokine treatment of established type II collagen-induced arthritis in DBA/1 mice. A comparitive study using anti-TNF-alpha, anti-IL1-alpha-beta, and IL1Ra. *Arthritis & Rheumatism*, **39,** 797-809.

Joyce, D.A., Gibbons, D.P., Green, P., Steer, J.H., Feldmann, M. & Brennan, F.M. (1994). Two inhibitors of pro-inflammatory cytokine release, interleukin-10 and interleukin-4, have contrasting effects on release of soluble p75 tumor necrosis factor receptor by cultured monocytes. *Eur J Immunol*, **24,** 2699-705.

Julius, D. (2004). The Molecular Biology of Thermosensation. In *Proceedings of the 10th World Congress on Pain*. eds Dostrovsky, J.O., Carr, D.B. & Koltzenburg, M. pp. 63-70. Seattle: IASP Press.

Julius, D. & McCleskey, E.W. (2006). Cellular and molecular poperties of primary afferent neurons, In *Textbook of Pain*. eds Wall, P.D. & Melzack, R. pp. 35-48. London: Churchill Livingstone.

Junger, H. & Sorkin, L.S. (2000). Nociceptive and inflammatory effects of subcutaneous TNFalpha. *Pain*, **85,** 145-51.

Kannan, K., Ortmann, R.A. & Kimpel, D. (2005). Animal models of rheumatoid arthritis and their relevance to human disease. *Pathophysiology*, **12,** 167-81.

Katsikis, P.D., Chu, C.Q., Brennan, F.M., Maini, R.N. & Feldmann, M. (1994). Immunoregulatory role of interleukin 10 in rheumatoid arthritis. *J Exp Med*, **179,** 1517-27.

Kavanaugh, A., St Clair, E.W., McCune, W.J., Braakman, T. & Lipsky, P. (2000). Chimeric anti-tumor necrosis factor-alpha monoclonal antibody treatment of patients with rheumatoid arthritis receiving methotrexate therapy. *J Rheumatol*, **27,** 841-50.

Kawaguchi, H., Pilbeam, C.C., Harrison, J.R. & Raisz, L.G. (1995). The role of prostaglandins in the regulation of bone metabolism. *Clin Orthop Relat Res*, 36-46.

Kazis, L.E., Meenan, R.F. & Anderson, J.J. (1983). Pain in the rheumatic diseases. Investigation of a key health status component. *Arthritis And Rheumatism*, **26,** 1017-1022.

Keeble, J., Blades, M., Pitzalis, C., Castro da Rocha, F.A. & Brain, S.D. (2005). The role of substance P in microvascular responses in murine joint inflammation. *Br J Pharmacol*, **144,** 1059-66.

Keeble, J.E., Curtis, B., Mallaghan, F.A. & Brain, S.D. (2004). The role of sensory nerves in joint inflammation: studies using TRPV1 knockout mice. *pA2 online*, **2,** 43P.

Keffer, J., Probert, L., Cazlaris, H., Georgopoulos, S., Kaslaris, E., Kioussis, D. & Kollias, G. (1991). Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. *Embo J*, **10,** 4025-31.

Kellenberger, S. and Schild, L. (2002). Epithelial sodium channels/degenerin family ion channels: a variety of functions for a shared structure. *Physiol Rev*, **82,** 735-67.

Kerr, B.J., Souslova, V., McMahon, S.B. & Wood, J.N. (2001). A role for the TTX-resistant sodium channel Nav 1.8 in NGF-induced hyperalgesia, but not neuropathic pain. *Neuroreport*, **12,** 3077-80.

Kester, M., Simonson, M.S., Mene, P. & Sedor, J.R. (1989). Interleukin-1 generates transmembrane signals from phospholipids through novel pathways in cultured rat mesangial cells. *J Clin Invest*, **83,** 718-23.

Khalsa, P.S., LaMotte, R.H. & Grigg, P. (1997). Tensile and compressive responses of nociceptors in rat hairy skin. *J Neurophysiol*, **78,** 492-505.

Khan, A.A., Raja, S.N., Manning, D.C., Campbell, J.N. & Meyer, R.A. (1992). The effects of bradykinin and sequence-related analogs on the response properties of cutaneous nociceptors in monkeys. *Somatosens Mot Res*, **9,** 97-106.

Khasar, S.G., Gold, M.S. & Levine, J.D. (1998). A tetrodotoxin-resistant sodium current mediates inflammatory pain in the rat. *Neurosci Lett*, **256,** 17-20.

Kim, B.M. & Oh, U. (2004). Inflammatory signals to TRPV1. In *Hyperalgesia: Molecular Mechanisms and Clinical Implications*. eds Brune, K. & Handwerker, H.O. pp. 27-36. Seattle: IASP Press.

Kirkham, B., Portek, I., Lee, C.S., Stavros, B., Lenarczyk, A., Lassere, M. & Edmonds, J. (1999). Intraarticular variability of synovial membrane histology, immunohistology, and cytokine mRNA expression in patients with rheumatoid arthritis. *J Rheumatol*, **26,** 777-84.

Kishimoto, T. (1992). Interleukin-6 and its receptor in autoimmunity. *J Autoimmun*, **5 Suppl A,** 123-32.

Kishimoto, T., Akira, S., Narazaki, M. & Taga, T. (1995). Interleukin-6 family of cytokines and gp130. *Blood*, **86,** 1243-54.

Knyihar-Csillik, E. & Csillik, B (1981). FRAP: histochemistry of the primary nociceptive neuron. *Prog Histochem Cytochem*, **14,** 1-137.

Kobayashi, I. & Ziff, M. (1975). Electron microscopic studies of the cartilage-pannus junction in rheumatoid arthritis. *Arthritis Rheum*, **18,** 475-83.

Kobayashi, K., Imaizumi, R., Sumichika, H., Tanaka, H., Goda, M., Fukunari, A. & Komatsu, H. (2003). Sodium iodoacetate-induced experimental osteoarthritis and associated pain model in rats. *J Vet Med Sci*, **65,** 1195-9.

Koch, A.E., Kunkel, S.L., Chensue, S.W., Haines, G.K. & Strieter, R.M. (1992a). Expression of interleukin-1 and interleukin-1 receptor antagonist by human rheumatoid synovial tissue macrophages. *Clin Immunol Immunopathol*, **65,** 23-9.

Koch, K.C., Ye, K., Clark, B.D. & Dinarello, C.A. (1992b). Interleukin 4 (IL) 4 up-regulates gene and surface IL 1 receptor type I in murine T helper type 2 cells. *Eur J Immunol*, **22,** 153-7.

Koda, H., Minagawa, M., Si-Hong, L., Mizumura, K. & Kumazawa, T. (1996). H1-receptor-mediated excitation and facilitation of the heat response by histamine in canine visceral polymodal receptors studied in vitro. *J Neurophysiol*, **76,** 1396-404.

Kohase, M., May, L.T., Tamm, I., Vilcek, J. & Sehgal, P.B. (1987). A cytokine network in human diploid fibroblasts: interactions of beta-interferons, tumor necrosis factor, platelet-derived growth factor, and interleukin-1. *Mol Cell Biol*, **7,** 273-80.

Koketsu, K. (1956). Intracellular potential changes of primary afferent nerve fibers in spinal cords of cats. *J Neurophysiol*, **19,** 375-92.

Koltzenburg, M. (2000). Neural mechanisms of cutaneous nociceptive pain. *Clin J Pain*, **16,** S131-8.

Kong, Y.Y., Yoshida, H., Sarosi, I., Tan, H.L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A.J., Van, G., Itie, A., Khoo, W., Wakeham, A., Dunstan, C.R., Lacey, D.L., Mak, T.W., Boyle, W.J. & Penninger, J.M. (1999). OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature*, **397,** 315-23.

Kontoyiannis, D., Pasparakis, M., Pizarro, T.T., Cominelli, F. and Kollias, G. (1999). Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity,* **10,** 387-98.

Korherr, C., Hofmeister, R., Wesche, H. & Falk, W. (1997). A critical role for interleukin-1 receptor accessory protein in interleukin-1 signaling. *Eur J Immunol*, **27,** 262-7.

Kosek, E., Ekholm, J. & Hansson, P. (1995). Increased pressure pain sensibility in fibromyalgia patients is located deep to the skin but not restricted to muscle tissue. *Pain*, **63,** 335-9.

Kotake, S., Sato, K., Kim, K.J., Takahashi, N., Udagawa, N., Nakamura, I., Yamaguchi, A., Kishimoto, T., Suda, T. & Kashiwazaki, S. (1996). Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. *J Bone Miner Res*, **11,** 88-95.

Kress, M., Koltzenburg, M., Reeh, P.W. & Handwerker, H.O. (1992). Responsiveness and functional attributes of electrically localized terminals of cutaneous C-fibers in vivo and in vitro. *J Neurophysiol*, **68,** 581-95.

Kress, M. & Reeh, P.W. (1996). Chemical excitation and sensitisation in nociceptors. In *Neurobiology of Nociceptors*. eds Belmonte, C. & Cervero, F. pp. 258-297. Oxford: Oxford University Press.

Ku, G., Ford, P., Raybuck, S.A., Harding, M.W. & randle, J.C.R. (2001). Selective interleukin-1 beta converting enzyme (ICE/caspase-1) inhibition with pralnacasan (HMR 4380/VX-740) reduces inflammation and joint destruction in murine type II collagen-induced arthritis (CIA). *Arthritis & Rheumatism*, **44,** S241.

Kubota, E., Kubota, T., Matsumoto, J., Shibata, T. & Murakami, K.I. (1998). Synovial fluid cytokines and proteinases as markers of temporomandibular joint disease. *J Oral Maxillofac Surg*, **56,** 192-8.

Kuenzi, F.M. & Dale, N. (1996). Effect of capsaicin and analogues on potassium and calcium currents and vanilloid receptors in Xenopus embryo spinal neurones. *Br J Pharmacol*, **119,** 81-90.

Kuiper, S., Joosten, L., Bendele, A., Edwards, C.K.I., Arntz, O.J., Helsen, M.M., van de Loo, A.A. & van den Berg, W. (1998). Different roles of TNF-alpha andinterleukin 1 in murine streptoccal cell wall arthritis. *Cytokine*, 690-702.

Kunicka, J.E., Talle, M.A., Denhardt, G.H., Brown, M., Prince, L.A. & Goldstein, G. (1993). Immunosuppression by glucocorticoids: inhibition of production of multiple lymphokines by in vivo administration of dexamethasone. *Cell Immunol*, **149,** 39-49.

Kwak, J.Y., Jung, J.Y., Hwang, S.W., Lee, W.T. & Oh, U. (1998). A capsaicin-receptor antagonist, capsazepine, reduces inflammation-induced hyperalgesic responses in the rat: evidence for an endogenous capsaicin-like substance. *Neuroscience*, **86,** 619-26.

Lader, C.S. & Flanagan, A.M. (1998). Prostaglandin E2, interleukin 1alpha, and tumor necrosis factor-alpha increase human osteoclast formation and bone resorption in vitro. *Endocrinology*, **139,** 3157-64.

LaMotte, R.H., Shain, C.N., Simone, D.A. & Tsai, E.F. (1991). Neurogenic hyperalgesia: psychophysical studies of underlying mechanisms. *J Neurophysiol*, **66,** 190-211.

Lang, E., Novak, A., Reeh, P.W. & Handwerker, H.O. (1990). Chemosensitivity of fine afferents from rat skin in vitro. *J Neurophysiol*, **63,** 887-901.

Langford, L.A. & Schmidt, R.F. (1983). Afferent and efferent axons in the medial and posterior articular nerves of the cat. *Anat Rec*, **206,** 71-8.

Langley, G.B., Fowles, M., Sheppeard, H. & Wigley, R.D. (1983). A simple pressure dolorimeter for the quantification of joint tenderness in inflammatory arthritis. *Rheumatol Int*, **3,** 109-12.

Langley, J.N. (1921). *The Autonomic Nervous System*. London: Heffner.

Lawand, N.B., Willis, W.D. & Westlund, K.N. (1997). Excitatory amino acid receptor involvement in peripheral nociceptive transmission in rats. *Eur J Pharmacol*, **324,** 169-77.

Lawrence, J.S. (1961). Prevalence of rheumatoid arthritis. *Ann Rheum Dis*, **20,** 11-7.

Le Bars, D., Gozariu, M. & Cadden, S.W. (2001). Animal models of nociception. *Pharmacol Rev*, **53,** 597-652.

Ledent, C., Vaugeois, J.M., Schiffmann, S.N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J.J., Costentin, J., Heath, J.K., Vassart, G. & Parmentier, M. (1997). Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature*, **388,** 674-8.

Lee, D.M., Friend, D.S., Gurish, M.F., Benoist, C., Mathis, D. & Brenner, M.B. (2002). Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science*, **297,** 1689-92.

Lee, D.M. & Weinblatt, M.E. (2001). Rheumatoid arthritis. *The Lancet*, **358,** 903-911.

Lee, P., Baxter, A., Dick, W.C. & Webb, J. (1974). An assessment of grip strength measurement in rheumatoid arthritis. *Scand J Rheumatol*, **3,** 17-23.

Lefkovits, I. & Waldmann, R. (1979). *Limiting dilution analysis of cells in the immune system*. Cambridge: Cambridge University Press.

Leisten, J.C., Gaarde, W.A. & Scholz, W. (1990). Interleukin-6 serum levels correlate with footpad swelling in adjuvant-induced arthritic Lewis rats treated with cyclosporin A or indomethacin. *Clin Immunol Immunopathol*, **56,** 108-15.

Lekan, H.A., Carlton, S.M. & Coggeshall, R.E. (1996). Sprouting of A beta fibers into lamina II of the rat dorsal horn in peripheral neuropathy. *Neurosci Lett*, **208,** 147-50.

Lemonnier, J., Hay, E., Delannoy, P., Fromigue, O., Lomri, A., Modrowski, D. & Marie, P.J. (2001). Increased osteoblast apoptosis in apert craniosynostosis: role of protein kinase C and interleukin-1. *Am J Pathol*, **158,** 1833-42.

Levine, J.D., Lau, W., Kwiat, G. & Goetzl, E.J. (1984). Leukotriene B4 produces hyperalgesia that is dependent on polymorphonuclear leukocytes. *Science*, **225,** 743-5.

Levine, J.D. & Tawoi, Y.O. (1994). Inflammatory Pain. In *Textbook of Pain*. eds Wall, P.D. & Melzack, R. pp. 45-56. London: Churchill Livingstone.

Lewinter, R.D., Skinner, D., Julius, D. & Basbaum, A.I. (2004). Immunoreactive TRPV-2 (VRL-1), a capsaicin receptor homolog in the spinal cord of the rat. *J Comp Neurol*, **470,** 400-408.

Lewis, C., Neidhart, S., Holy, C., North, R.A., Buell, G. & Surprenant, A. (1995). Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. *Nature*, **377,** 432-5.

Lewis, T. (1942). *Pain*. New York: macmillan.

Lewthwaite, J., Blake, S., Hardingham, T., Foulkes, R., Stephens, S., Chaplin, L., Emtage, S., Catterall, C., Short, S., Nesbitt, A. & et al. (1995). Role of TNF alpha in the induction of antigen induced arthritis in the rabbit and the anti-arthritic effect of species specific TNF alpha neutralising monoclonal antibodies. *Ann Rheum Dis*, **54,** 366-74.

Li, W. & Neugebauer, V. (2004). Differential roles of mGluR1 and mGluR5 in brief and prolonged nociceptive processing in central amygdala neurons. *J Neurophysiol*, **91,** 13-24.

Littlewood, A.J., Russell, J., Harvey, G.R., Hughes, D.E., Russell, R.G. & Gowen, M. (1991). The modulation of the expression of IL-6 and its receptor in human osteoblasts in vitro. *Endocrinology*, **129,** 1513-20.

Liu, L. & Simon, S.A. (1997). Capsazepine, a vanilloid receptor antagonist, inhibits nicotinic acetylcholine receptors in rat trigeminal ganglia. *Neurosci Lett*, **228,** 29-32.

Loher, F., Bauer, C., Landauer, N., Schmall, K., Siegmund, B., Lehr, H.A., Dauer, M., Schoenharting, M., Endres, S. & Eigler, A. (2004). The interleukin-1 beta-converting enzyme inhibitor pralnacasan reduces dextran sulfate sodium-induced murine colitis and T helper 1 T-cell activation. *J Pharmacol Exp Ther*, **308,** 583-90.

Lorenz, H.M., Grunke, M., Hieronymus, T., Antoni, C., Nusslein, H., Schaible, T.F., Manger, B. & Kalden, J.R. (2000). In vivo blockade of tumor necrosis factor-alpha in patients with rheumatoid arthritis: longterm effects after repeated infusion of chimeric monoclonal antibody cA2. *J Rheumatol*, **27,** 304-10.

Luis-Delgado, O.E., Barrot, M., Rodeau, J.L., Schott, G., Benbouzid, M., Poisbeau, P., Freund-Mercier, M.J. & Lasbennes, F. (2006). Calibrated forceps: a sensitive and reliable tool for pain and analgesia studies. *J Pain*, **7,** 32-9.

Ma, Q.P. (2001). Vanilloid receptor homologue, VRL1, is expressed by both A- and C-fibre sensory neurons. *Neuroreport*, **12,** 3693-3695.

Magari, K., Miyata, S., Nishigaki, F., Ohkubo, Y., Mutoh, S. & Goto, T. (2003). Differential effects of FK506 and methotrexate on inflammatory cytokine levels in rat adjuvant-induced arthritis. *J Rheumatol*, **30,** 2193-200.

Maggi, C.A. (1995). Tachykinins and calcitonin gene-related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves. *Prog Neurobiol*, **45,** 1-98.

Maini, R., Paulus, H. & Breedveld, F. (1997). rHuIL-10 in subjects with active rheumatoid arthritis (RA): a phase I and cytokine response study. *Arthritis & Rheumatism*, **40,** S224.

Maini, R.N. & Taylor, P.C. (2000). Anti-cytokine therapy for rheumatoid arthritis. *Annu Rev Med*, **51,** 207-29.

Maini, R.N., Taylor, P.C., Szechinski, J., Pavelka, K., Broll, J., Balint, G., Emery, P., Raemen, F., Petersen, J., Smolen, J., Thomson, D. & Kishimoto, T. (2006). Double-blind randomized controlled clinical trial of the interleukin-6 receptor antagonist, tocilizumab, in European patients with rheumatoid arthritis who had an incomplete response to methotrexate. *Arthritis Rheum*, **54,** 2817-29.

Mancilla, J., Ikejima, T. & Dinarello, C.A. (1992). Glycosylation of the interleukin-1 receptor type I is required for optimal binding of interleukin-1. *Lymphokine Cytokine Res*, **11,** 197-205.

Manning, D.C., Raja, S.N., Meyer, R.A. & Campbell, J.N. (1991). Pain and hyperalgesia after intradermal injection of bradykinin in humans. *Clin Pharmacol Ther*, **50,** 721-9.

Mantyselka, P., Kumpusalo, E., Ahonen, R., Kumpusalo, A., Kauhanen, J., Viinamaki, H., Halonen, P. & Takala, J. (2001). Pain as a reason to visit the doctor: a study in Finnish primary health care. *Pain*, **89,** 175-80.

Mapp, P.I. (1995). Innervation of the synovium. *Ann Rheum Dis*, **54,** 398-403.

Mapp, P.I., Grootveld, M.C. & Blake, D.R. (1995). Hypoxia, oxidative stress and rheumatoid arthritis. *Br Med Bull*, **51,** 419-36.

Mapp, P.I., Terenghi, G., Walsh, D.A., Chen, S.T., Cruwys, S.C., Garrett, N., Kidd, B.L., Polak, J.M. & Blake, D.R. (1993). Monoarthritis in the rat knee induces bilateral and time-dependent changes in substance P and calcitonin gene-related peptide immunoreactivity in the spinal cord. *Neuroscience*, **57,** 1091-6.

Marinova-Mutafchieva, L., Williams, R.O., Mason, L.J., Mauri, C., Feldmann, M. & Maini, R.N. (1997). Dynamics of proinflammatory cytokine expression in the joints of mice with collagen-induced arthritis (CIA). *Clin Exp Immunol*, **107,** 507-12.

Martin, H.A., Basbaum, A.I., Kwiat, G.C., Goetzl, E.J. & Levine, J.D. (1987). Leukotriene and prostaglandin sensitization of cutaneous high-threshold C- and A-delta mechanonociceptors in the hairy skin of rat hindlimbs. *Neuroscience*, **22,** 651-9.

Martin, M.U. & Falk, W. (1997). The interleukin-1 receptor complex and interleukin-1 signal transduction. *Eur Cytokine Netw*, **8,** 5-17.

Matsumoto, T., Tsurumoto, T. & Shindo, H. (2006). Interleukin-6 levels in synovial fluid of patients with rheumatoid arthritis correlated with the infiltration of inflammatory cells in synovial membrane. *Rheumatol Int.*, **26,** 1096-1100.

Matteson, E. & Mason, T. (2005). *Atlas of Rheumatology*. UK: Blackwell Science.

McKemy, D.D., Neuhausser, W.M. & Julius, D. (2002). Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature*, **416,** 52-8.

McKenna, F. & Wright, V. (1985). Pain and rheumatoid arthritis. *Annals Of The Rheumatic Diseases*, **44,** 805.

McKenna, R.M., Ofosu-Appiah, W., Warrington, R.J. & Wilkins, J.A. (1986). Interleukin 2 production and responsiveness in active and inactive rheumatoid arthritis. *J Rheumatol*, **13,** 28-32.

McLatchie, L.M. & Bevan, S. (2001). The effects of pH on the interaction between capsaicin and the vanilloid receptor in rat dorsal root ganglia neurons. *Br J Pharmacol*, **132,** 899-908.

McMahan, C.J., Slack, J.L., Mosley, B., Cosman, D., Lupton, S.D., Brunton, L.L., Grubin, C.E., Wignall, J.M., Jenkins, N.A., Brannan, C.I. & et al. (1991). A novel IL-1 receptor,

cloned from B cells by mammalian expression, is expressed in many cell types. *Embo J*, **10,** 2821-32.

McMurdo, L., Lockhart, J.C. & Ferrell, W.R. (1997). Modulation of synovial blood flow by the calcitonin gene-related peptide (CGRP) receptor antagonist, CGRP(8-37). *Br J Pharmacol*, **121,** 1075-80.

McNiff, P.A., Stewart, C., Sullivan, J., Showell, H.J. & Gabel, C.A. (1995). Synovial fluid from rheumatoid arthritis patients contains sufficient levels of IL1β and IL6 to promote production of serum amyloid A by HEP3B cells. *Cytokine,* **7,** 209-219.

McQuay, H. & Moore, A. (2002). TNF antibodies and rheumatoid arthritis. In *Bandolier - Evidenve Based Health Care.*

Meenan, R.F., Gertman, P.M. & Mason, J.H. (1980). Measuring health status in arthritis. The arthritis impact measurement scales. *Arthritis Rheum*, **23,** 146-52.

Melzack, R. (1975). The McGill Pain Questionnaire: major properties and scoring methods. *Pain*, **1,** 277-99.

Mendell, L.M. (1966). Physiological properties of unmyelinated fiber projection to the spinal cord. *Exp Neurol*, **16,** 316-32.

Mense, S. & Meyer, H. (1985). Different types of slowly conducting afferent units in cat skeletal muscle and tendon. *J Physiol*, **363,** 403-17.

Merskey, H. & Bogduk, N. (1994). *Classification of chronic pain: descriptions of chronic pain syndromes and definition of pain terms.* Seattle: IASP Press.

Meyer, F.A., Yaron, I. & Yaron, M. (1990). Synergistic, additive, and antagonistic effects of interleukin-1 beta, tumor necrosis factor alpha, and gamma-interferon on prostaglandin E, hyaluronic acid, and collagenase production by cultured synovial fibroblasts. *Arthritis Rheum*, **33,** 1518-25.

Michael, G.J. & Priestley, J.V. (1999). Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. *J Neurosci*, **19,** 1844-54.

Millan, M.J. (1999). The induction of pain: an integrative review. *Prog Neurobiol*, **57,** 1-164.

Min, S.S., Han, J.S., Kim, Y.I., Na, H.S., Yoon, Y.W., Hong, S.K. & Han, H.C. (2001). A novel method for convenient assessment of arthritic pain in voluntarily walking rats. *Neurosci Lett*, **308,** 95-8.

Miossec, P., Naviliat, M., Dupuy d'Angeac, A., Sany, J. & Banchereau, J. (1990). Low levels of interleukin-4 and high levels of transforming growth factor beta in rheumatoid synovitis. *Arthritis Rheum*, **33,** 1180-7.

Miyata, M., Ohira, H., Sasajima, T., Suzuki, S., Ito, M., Sato, Y. & Kasukawa, R. (2000). Significance of low mRNA levels of interleukin-4 and -10 in mononuclear cells of the synovial fluid of patients with rheumatoid arthritis. *Clin Rheumatol*, **19,** 365-70.

Mizel, S.B. (1990). How does interleukin 1 activate cells? Cyclic AMP and interleukin 1 signal transduction. *Immunol Today*, **11,** 390-1.

Mizel, S.B., Dayer, J.M., Krane, S.M. & Mergenhagen, S.E. (1981). Stimulation of rheumatoid synovial cell collagenase and prostaglandin production by partially purified lymphocyte-activating factor (interleukin 1). *Proc Natl Acad Sci U S A*, **78,** 2474-7.

Mizumura, K., Minagawa, M., Koda, H. & Kumazawa, T. (1995). Influence of histamine on the bradykinin response of canine testicular polymodal receptors in vitro. *Inflamm Res*, **44,** 376-8.

Mohr, W. & Menninger, H. (1980). Polymorphonuclear granulocytes at the pannus-cartilage junction in rheumatoid arthritis. *Arthritis Rheum*, **23,** 1413-14.

Molliver, D.C., Radeke, M.J., Feinstein, S.C. & Snider, W.D. (1995). Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. *J Comp Neurol*, **361,** 404-16.

Moos, V., Fickert, S., Muller, B., Weber, U. & Sieper, J. (1999). Immunohistological analysis of cytokine expression in human osteoarthritic and healthy cartilage. *J Rheumatol*, **26,** 870-9.

Moreland, L.W., McCabe, D.P., Caldwell, J.R., Sack, M., Weisman, M., Henry, G., Seely, J.E., Martin, S.W., Yee, C.L., Bendele, A.M., Frazier, J.L., Kohno, T., Cosenza, M.E., Lyons, S.A., Dayer, J.M., Cohen, A.M. & Edwards, C.K., 3rd (2000). Phase I/II trial of recombinant methionyl human tumor necrosis factor binding protein PEGylated dimer in patients with active refractory rheumatoid arthritis. *J Rheumatol*, **27,** 601-9.

Mullberg, J., Schooltink, H., Stoyan, T., Gunther, M., Graeve, L., Buse, G., Mackiewicz, A., Heinrich, P.C. & Rose-John, S. (1993). The soluble interleukin-6 receptor is generated by shedding. *Eur J Immunol*, **23,** 473-80.

Muller-Ladner, U., Gay, R.E. & Gay, S. (1997). Cellular pathways of joint destruction. *Curr Opin Rheumatol*, **9,** 213-20.

Murphy, P.G., Ramer, M.S., Borthwick, L., Gauldie, J., Richardson, P.M. & Bisby, M.A. (1999). Endogenous interleukin-6 contributes to hypersensitivity to cutaneous stimuli and changes in neuropeptides associated with chronic nerve constriction in mice. *Eur J Neurosci*, **11,** 2243-53.

Myers, L.K., Rosloniec, E.F., Cremer, M.A. & Kang, A.H. (1997). Collagen-induced arthritis, an animal model of autoimmunity. *Life Sci*, **61,** 1861-78.

Nagy, I. & Rang, H. (1999). Noxious heat activates all capsaicin-sensitive and also a sub-population of capsaicin-insensitive dorsal root ganglion neurons. *Neuroscience*, **88,** 995-7.

Nakamura, F. & Strittmatter, S.M. (1996). P2Y1 purinergic receptors in sensory neurons: contribution to touch-induced impulse generation. *Proc Natl Acad Sci U S A*, **93,** 10465-70.

Nakamura, H., Motoyoshi, S. & Kadokawa, T. (1988). Anti-inflammatory action of interleukin 1 through the pituitary-adrenal axis in rats. *Eur J Pharmacol*, **151,** 67-73.

Nassar, M.A., Stirling, L.C., Forlani, G., Baker, M.D., Matthews, E.A., Dickenson, A.H. & Wood, J.N. (2004). Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain. *Proc Natl Acad Sci U S A*, **101,** 12706-11.

Neidel, J., Schulze, M. & Lindschau, J. (1995). Association between degree of bone-erosion and synovial fluid-levels of tumor necrosis factor alpha in the knee-joints of patients with rheumatoid arthritis. *Inflamm Res*, **44,** 217-21.

Nelson, E.K. (1991). The constitution of capsaicin,
the pungent principal of capsicum. *J. Am. Chem. Soc.*, **41,** 1115-1121.

Neugebauer, V. & Li, W. (2002). Processing of nociceptive mechanical and thermal information in central amygdala neurons with knee-joint input. *J Neurophysiol*, **87,** 103-12.

Niki, Y., Yamada, H., Kikuchi, T., Toyama, Y., Matsumoto, H., Fujikawa, K. & Tada, N. (2004). Membrane-associated IL-1 contributes to chronic synovitis and cartilage destruction in human IL-1 alpha transgenic mice. *J Immunol*, **172,** 577-84.

Niki, Y., Yamada, H., Seki, S., Kikuchi, T., Takaishi, H., Toyama, Y., Fujikawa, K. and Tada, N. (2001). Macrophage- and neutrophil-dominant arthritis in human IL1α transgenic mice. *J CLin invest,* **107,** 1127-35.

Nordan, R.P. & Potter, M. (1986). A macrophage-derived factor required by plasmacytomas for survival and proliferation in vitro. *Science*, **233,** 566-9.

Nordlind, K. & Vahlquist, A. (1999). [New trends in immunodermatology and treatment of skin diseases]. *Lakartidningen*, **96,** 876-81.

Notoya, K., Jovanovic, D.V., Reboul, P., Martel-Pelletier, J., Mineau, F. & Pelletier, J.P. (2000). The induction of cell death in human osteoarthritis chondrocytes by nitric oxide is related to the production of prostaglandin E2 via the induction of cyclooxygenase-2. *J Immunol*, **165,** 3402-10.

Obreja, O., Rathee, P.K., Lips, K.S., Distler, C. & Kress, M. (2002). IL-1 beta potentiates heat-activated currents in rat sensory neurons: involvement of IL-1RI, tyrosine kinase, and protein kinase C. *FASEB J,* **16,** 1497-503.

Obreja, O., Schmelz, M., Poole, S. & Kress, M. Interleukin-6 in combination with its soluble IL-6 receptor sensitizes rat skin nociceptors to heat in vivo. *Pain,* **96,** 57-62.

Ohshima, S., Saeki, Y., Mima, T., Sasai, M., Nishioka, K., Nomura, S., Kopf, M., Katada, Y., Tanaka, T., Suemura, M. & Kishimoto, T. (1998). Interleukin 6 plays a key role in the development of antigen-induced arthritis. *Proc Natl Acad Sci U S A*, **95,** 8222-6.

Oka, T., Oka, K., Hosoi, M. & Hori, T. (1995). Intracerebroventricular injection of interleukin-6 induces thermal hyperalgesia in rats. *Brain Res*, **692,** 123-8.

Okada, M., Kitahara, M., Kishimoto, S., Matsuda, T., Hirano, T. & Kishimoto, T. (1988). IL-6/BSF-2 functions as a killer helper factor in the in vitro induction of cytotoxic T cells. *J Immunol*, **141,** 1543-9.

O'Neill, L.A., Bird, T.A., Gearing, A.J. & Saklatvala, J. (1990). Interleukin-1 signal transduction. Increased GTP binding and hydrolysis in membranes of a murine thymoma line (EL4). *J Biol Chem*, **265,** 3146-52.

Opree, A. & Kress, M. (2000). Involvement of the proinflammatory cytokines tumor necrosis factor-alpha, IL-1 beta, and IL-6 but not IL-8 in the development of heat hyperalgesia: effects on heat-evoked calcitonin gene-related peptide release from rat skin. *J Neurosci*, **20,** 6289-93.

Orozco, O.E., Walus, L., Sah, D.W., Pepinsky, R.B. & Sanicola, M. (2001). GFRalpha3 is expressed predominantly in nociceptive sensory neurons. *Eur J Neurosci*, **13,** 2177-82.

Otsuki, T., Nakahama, H., Niizuma, H. & Suzuki, J. (1986). Evaluation of the analgesic effects of capsaicin using a new rat model for tonic pain. *Brain Res*, **365,** 235-40.

Ottillinger, B., Gomor, B., Michel, B.A., Pavelka, K., Beck, W. & Elsasser, U. (2001). Efficacy and safety of eltenac gel in the treatment of knee osteoarthritis. *Osteoarthritis Cartilage*, **9,** 273-80.

Ozaki, M., Kawabe, Y., Nakamura, H., Migita, K., Kawakami, A., Tsukazaki, K. & Eguchi, K. (2001). Elevated serum cytokine levels in a rheumatoid arthritis patient with large granular lymphocyte syndrome. *Rheumatology (Oxford)*, **40,** 592-3.

Ozaktay, A.C., Kallakuri, S., Takebayashi, T. Cavanaugh, J.M., Asik, I., DeLeo, J.A. & Weinstein, J.N. (2006). Effects of interleukin-1 beta, interleukin-6 and tumour necrosis factor on sensitivity of dorsal root ganglion and peripheral receptive fields in rats. *Eur J Spine,* **15,** 1529-1537.

Panzer, S., Madden, M. & Matsuki, K. (1993). Interaction of IL-1 beta, IL-6 and tumour necrosis factor-alpha (TNF-alpha) in human T cells activated by murine antigens. *Clin Exp Immunol*, **93,** 471-8.

Partsch, G., Schwagerl, W. & Eberl, R. (1982). [Histamine in rheumatic diseases]. *Z Rheumatol*, **41,** 19-22.

Patten, C., Bush, K., Rioja, I., Morgan, R., Wooley, P., Trill, J. & Life, P. (2004). Characterization of pristane-induced arthritis, a murine model of chronic disease: response to antirheumatic agents, expression of joint cytokines, and immunopathology. *Arthritis Rheum*, **50,** 3334-45.

Pearson, C.M. (1956). Development of arthritis, periarthritis and perioscitis in rats given adjuvants. *Proc Soc Exp Biol Med*, **91,** 95-101.

Pearson, C.M. & Wood, F.D. (1959). Studies of polyarthritis and other lesions induced in rats given adjuvants. *Arthritis and Rheumatism*, **2,** 440-459.

Peier, A.M., Moqrich, A., Hergarden, A.C., Reeve, A.J., Andersson, D.A., Story, G.M., Earley, T.J., Dragoni, I., McIntyre, P., Bevan, S. & Patapoutian, A. (2002a). A TRP channel that senses cold stimuli and menthol. *Cell*, **108,** 705-15.

Peier, A.M., Reeve, A.J., Andersson, D.A., Moqrich, A., Earley, T.J., Hergarden, A.C., Story, G.M., Colley, S., Hogenesch, J.B., McIntyre, P., Bevan, S. & Patapoutian, A. (2002b). A heat-sensitive TRP channel expressed in keratinocytes. *Science*, **296,** 2046-9.

Pelegri, C., Franch, A., Castellote, C. & Castell, M. (1995). Immunohistochemical changes in synovial tissue during the course of adjuvant arthritis. *J Rheumatol*, **22,** 124-32.

Pettipher, E.R., Higgs, G.A. & Henderson, B. (1986). Interleukin 1 induces leukocyte infiltration and cartilage proteoglycan degradation in the synovial joint. *Proc Natl Acad Sci U S A*, **83,** 8749-53.

Pierau, F.K., Torrey, P. & Carpenter, D.O. (1974). Mammalian cold receptor afferents: role of an electrogenic sodium pump in sensory transduction. *Brain Res*, **73,** 156-60.

Pignatti, P., Ciapponi, L., Galle, P., Hansen, M.B., Massa, M., Meazza, C., Paonessa, G., Novick, D., Ciliberto, G., Martini, A. & De Benedetti, F. (2003). High circulating levels of biologically inactive IL-6/SIL-6 receptor complexes in systemic juvenile idiopathic arthritis: evidence for serum factors interfering with the binding to gp130. *Clin Exp Immunol*, **131,** 355-63.

Pincus, T., Brooks, R.H. & Callahan, L.F. (1994). Prediction of long-term mortality in patients with rheumatoid arthritis according to simple questionnaire and joint count measures. *Ann Intern Med*, **120,** 26-34.

Pincus, T. & Callahan, L.F. (1992). Rheumatology function tests: grip strength, walking time, button test and questionnaires document and predict longterm morbidity and mortality in rheumatoid arthritis. *J Rheumatol*, **19,** 1051-7.

Pincus, T., Callahan, L.F., Sale, W.G., Brooks, A.L., Payne, L.E. & Vaughn, W.K. (1984). Severe functional declines, work disability, and increased mortality in seventy-five rheumatoid arthritis patients studied over nine years. *Arthritis Rheum*, **27,** 864-72.

Pincus, T., Callahan, L.F. & Vaughn, W.K. (1987). Questionnaire, walking time and button test measures of functional capacity as predictive markers for mortality in rheumatoid arthritis. *J Rheumatol*, **14,** 240-51.

Pinheiro, R.M. & Calixto, J.B. (2002). Effect of the selective COX-2 inhibitors, celecoxib and rofecoxib in rat acute models of inflammation. *Inflam Res,* **51,** 603-10.

Plaut, M., Pierce, J.H., Watson, C.J., Hanley-Hyde, J., Nordan, R.P. & Paul, W.E. (1989). Mast cell lines produce lymphokines in response to cross-linkage of Fc epsilon RI or to calcium ionophores. *Nature*, **339,** 64-7.

Poole, S., Cunha, F. & Ferreira, S.H. (1999). Hyperalgesia from subcutaneous cytokines. In *Cyokines and Pain.* eds Watkins, L.R. & Maier, S.F. pp. 59-87. Basel: Birkhauser.

Porreca, F., Lai, J., Bian, D., Wegert, S., Ossipov, M.H., Eglen, R.M., Kassotakis, L., Novakovic, S., Rabert, D.K., Sangameswaran, L. & Hunter, J.C. (1999). A comparison of the potential role of the tetrodotoxin-insensitive sodium channels, PN3/SNS and NaN/SNS2, in rat models of chronic pain. *Proc Natl Acad Sci U S A*, **96,** 7640-4.

Priestle, J.P., Schar, H.P. & Grutter, M.G. (1989). Crystallographic refinement of interleukin 1 beta at 2.0 A resolution. *Proc Natl Acad Sci U S A*, **86,** 9667-71.

Pyne, D., Ioannou, Y., Mootoo, R. & Bhanji, A. (2004). Intra-articular steroids in knee osteoarthritis: a comparative study of triamcinolone hexacetonide and methylprednisolone acetate. *Clin Rheumatol*, **23,** 116-20.

Ralph, P., Nakoinz, I., Sampson-Johannes, A., Fong, S., Lowe, D., Min, H.Y. & Lin, L. (1992). IL-10, T lymphocyte inhibitor of human blood cell production of IL-1 and tumor necrosis factor. *J Immunol*, **148,** 808-14.

Randall, L.O. & Selitto, J.J. (1957). A method for measurement of analgesic activity on inflamed tissue. *Arch Int Pharmacodyn Ther*, **111,** 409-19.

Rathanaswami, P., Hachicha, M., Wong, W.L., Schall, T.J. & McColl, S.R. (1993). Synergistic effect of interleukin-1 beta and tumor necrosis factor alpha on interleukin-8 gene expression in synovial fibroblasts. Evidence that interleukin-8 is the major neutrophil-activating chemokine released in response to monokine activation. *Arthritis Rheum*, **36,** 1295-304.

Re, F., Muzio, M., De Rossi, M., Polentarutti, N., Giri, J.G., Mantovani, A. & Colotta, F. (1994). The type II "receptor" as a decoy target for interleukin 1 in polymorphonuclear leukocytes: characterization of induction by dexamethasone and ligand binding properties of the released decoy receptor. *J Exp Med*, **179,** 739-43.

Reeh, P.W. & Sauer, S.K. (1997). *Chronic aspects in peripheral nociception.* Seattle: IASP Press.

Reichert, F., Levitzky, R. & Rotshenker, S. (1996). Interleukin 6 in intact and injured mouse peripheral nerves. *Eur J Neurosci*, **8,** 530-5.

Reid, G. (2005). ThermoTRP channels and cold sensing: what are they really up to? *Pflug Arch – Eur J Physiol*, **451,** 250-263.

Reid, G. & Flonta, M. (2001a). Cold transduction by inhibition of a background potassium conductance in rat primary sensory neurones. *Neurosci Lett*, **297,** 171-4.

Reid, G. & Flonta, M.L. (2001b). Physiology. Cold current in thermoreceptive neurons. *Nature*, **413,** 480.

Rekola, K.E., Keinanen-Kiukaanniemi, S. & Takala, J. (1993). Use of primary health services in sparsely populated country districts by patients with musculoskeletal symptoms: consultations with a physician. *J Epidemiol Community Health*, **47,** 153-7.

Rexed, B. (1952). The cytoarchitectonic organization of the spinal cord in the cat. *J Comp Neurol.* **96**, 414-95.

Rice, A.S., Farquhar-Smith, W.P. & Nagy, I. (2002). Endocannabinoids and pain: spinal and peripheral analgesia in inflammation and neuropathy. *Prostaglandins Leukot Essent Fatty Acids*, **66,** 243-56.

Richardson, B.P. & Engel, G. (1986). The Pharmacology and function of 5-HT3 receptors. *Trends in Neurosciences*, **9,** 424-427.

Rickard, D.J. & Gowen, M. (1993). Cytokines in Arthritis. *Reports on Rheumatic Diseases*, **24**.

Rioja, I., Bush, K.A., Buckton, J.B., Dickson, M.C. & Life, P.F. (2004). Joint cytokine quantification in two rodent arthritis models: kinetics of expression, correlation of mRNA and protein levels and response to prednisolone treatment. *Clin Exp Immunol*, **137,** 65-73.

Ritchie, D.M., Boyle, J.A., McInnes, J.M., Jasani, M.K., Dalakos, T.G., Grieveson, P. & Buchanan, W.W. (1968). Clinical studies with an articular index for the assessment of joint tenderness in patients with rheumatoid arthritis. *Q J Med*, **37,** 393-406.

Robinson, D.R., Smith, H., McGuire, M.B. & Levine, L. (1975a). Prostaglandin synthesis by rheumatoid synovium and its stimulation by colchicine. *Prostaglandins*, **10,** 67-85.

Robinson, D.R., Tashjian, A.H., Jr. & Levine, L. (1975b). Prostaglandin-stimulated bone resorption by rheumatoid synovia. A possible mechanism for bone destruction in rheumatoid arthritis. *J Clin Invest*, **56,** 1181-8.

Roodman, G.D., Kurihara, N., Ohsaki, Y., Kukita, A., Hosking, D., Demulder, A., Smith, J.F. & Singer, F.R. (1992). Interleukin 6. A potential autocrine/paracrine factor in Paget's disease of bone. *J Clin Invest*, **89,** 46-52.

Rooney, M., Symons, J.A. & Duff, G.W. (1990). Interleukin 1 beta in synovial fluid is related to local disease activity in rheumatoid arthritis. *Rheumatol Int*, **10,** 217-9.

Rosenberg, A. (1999). Bones, Joints and Soft Tussue Tumours. In *Pathologic Basis of Disease*. eds Cotran, R.S., Kumar, V. & Collins, T.C. pp. 1216-1259. London: W.B.Saunders Company.

Rosenburg, A. (1999). Bones, Joints and Soft Tissue Tumours. In *Pathologic Basis of Disease*. eds Cotran, R.S., Kumar, V. & Collins, T. London: W.B. Saunders Company.

Rosoff, P.M., Savage, N. & Dinarello, C.A. (1988). Interleukin-1 stimulates diacylglycerol production in T lymphocytes by a novel mechanism. *Cell*, **54,** 73-81.

Rudolphi, K., Gerwin, N., Verzijl, N., van der Kraan, P. & van den Berg, W. (2003). Pralnacasan, an inhibitor of interleukin-1beta converting enzyme, reduces joint damage in two murine models of osteoarthritis. *Osteoarthritis Cartilage*, **11,** 738-46.

Saha, N., Moldovan, F., Tardif, G., Pelletier, J.P., Cloutier, J.M. & Martel-Pelletier, J. (1999). Interleukin-1beta-converting enzyme/caspase-1 in human osteoarthritic tissues: localization and role in the maturation of interleukin-1beta and interleukin-18. *Arthritis Rheum*, **42,** 1577-87.

Sakaguchi, S. & Sakaguchi, N. (2005). Animal models of arthritis caused by systemic alteration of the immune system. *Curr Opin Immunol*, **17,** 589-594

Samad, T.A., Moore, K.A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Bonventre, J.V. & Woolf, C.J. (2001). Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature*, **410,** 471-5.

Samuelsson, B. (1983). Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science*, **220,** 568-75.

Sanceau, J., Beranger, F., Gaudelet, C. & Wietzerbin, J. (1989). IFN-gamma is an essential cosignal for triggering IFN-beta 2/BSF-2/IL-6 gene expression in human monocytic cell lines. *Ann N Y Acad Sci*, **557,** 130-41, discussion 141-3.

Sano, H., Hla, T., Maier, J.A., Crofford, L.J., Case, J.P., Maciag, T. & Wilder, R.L. (1992). In vivo cyclooxygenase expression in synovial tissues of patients with rheumatoid arthritis and osteoarthritis and rats with adjuvant and streptococcal cell wall arthritis. *J Clin Invest*, **89,** 97-108.

Santos, L. & Tipping, P.G. (1994). Attenuation of adjuvant arthritis in rats by treatment with oxygen radical scavengers. *Immunol Cell Biol*, **72,** 406-14.

Sapru, H.N. & Krieger, A.J. (1979). Cardiovascular and respiratory effects of some anesthetics in the decerebrate rat. *Eur J Pharmacol*, **53,** 151-8.

Sawin, E.R., Ranganathan, R. & Horitz, H.R. (2000). C.elegans locomotory role is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. *Neuron*, **26,** 619-31.

Sawynok, J. (1998). Adenosine receptor activation and nociception. *Eur J Pharmacol*, **347,** 1-11.

Sawynok, J. & Reid, A. (1997). Peripheral adenosine 5'-triphosphate enhances nociception in the formalin test via activation of a purinergic p2X receptor. *Eur J Pharmacol*, **330,** 115-21.

Saxne, T., Palladino, M.A., Jr., Heinegard, D., Talal, N. & Wollheim, F.A. (1988). Detection of tumor necrosis factor alpha but not tumor necrosis factor beta in rheumatoid arthritis synovial fluid and serum. *Arthritis Rheum*, **31,** 1041-5.

Schaible, H.G., Ebersberger, A. & Von Banchet, G.S. (2002). Mechanisms of pain in arthritis. *Ann N Y Acad Sci*, **966,** 343-54.

Schaible, H.G. & Grubb, B.D. (1993). Afferent and spinal mechanisms of joint pain. *Pain*, **55,** 5-54.

Schaible, H.G. & Schmidt, R.F. (1983a). Activation of groups III and IV sensory units in medial articular nerve by local mechanical stimulation of knee joint. *J Neurophysiol*, **49,** 35-44.

Schaible, H.G. & Schmidt, R.F. (1985). Effects of an experimental arthritis on the sensory properties of fine articular afferent units. *J Neurophysiol*, **54,** 1109-22.

Schaible, H.G. & Schmidt, R.F. (1983b). Responses of fine medial articular nerve afferents to passive movements of knee joints. *J Neurophysiol*, **49,** 1118-26.

Schaible, H.G. & Schmidt, R.F. (1988). Time course of mechanosensitivity changes in articular afferents during a developing experimental arthritis. *J Neurophysiol*, **60,** 2180-95.

Schindler, R., Ghezzi, P. & Dinarello, C.A. (1990). IL-1 induces IL-1. IV. IFN-gamma suppresses IL-1 but not lipopolysaccharide-induced transcription of IL-1. *J Immunol*, **144,** 2216-22.

Schmelz, M., Schmidt, R., Bickel, A., Handwerker, H.O. & Torebjork, H.E. (1997). Specific C-receptors for itch in human skin. *J Neurosci*, **17,** 8003-8.

Schmelz, M., Schmidt, R., Weidner, C., Hilliges, M., Torebjork, H.E. & Handwerker, H.O. (2003). Chemical response pattern of different classes of C-nociceptors to pruritogens and algogens. *J Neurophysiol*, **89,** 2441-8.

Schmidt-Weber, C.B., Pohlers, D., Siegling, A., Schadlich, H., Buchner, E., Volk, H.D., Palombo-Kinne, E., Emmrich, F. & Kinne, R.W. (1999). Cytokine gene activation in synovial membrane, regional lymph nodes, and spleen during the course of rat adjuvant arthritis. *Cell Immunol*, **195,** 53-65.

Schnitzer, T.J., Weaver, A.L., Polis, A.B., Petruschke, R.A. & Geba, G.P. (2005). Efficacy of rofecoxib, celecoxib, and acetaminophen in patients with osteoarthritis of the knee. A combined analysis of the VACT studies. *J Rheumatol*, **32,** 1093-105.

Schott, E., Berge, O.G., Angeby-Moller, K., Hammarstrom, G., Dalsgaard, C.J. & Brodin, E. (1994). Weight bearing as an objective measure of arthritic pain in the rat. *J Pharmacol Toxicol Methods*, **31,** 79-83.

Schwab, J.H. (1995). Bacterial cell-wall induced arthritis: models of chronic recurrent polyarthritis and reactivation of monoarticular arthritis. In *Mechanisms and Models of Rheumatoid arthritis*. ed Pettipher, E.R. pp. 431-446. San Diego: Academic Press.

Schwab, J.H., Anderle, S.K., Brown, R.R., Dalldorf, F.G. & Thompson, R.C. (1991). Pro- and anti-inflammatory roles of interleukin-1 in recurrence of bacterial cell wall-induced arthritis in rats. *Infect Immun*, **59,** 4436-42.

Scott, D.L. (2006). Osteoarthritis and Rheumatoid Arthritis. In *Wall and Melzack's Textbook of Pain*. eds McMahon, S.B. & Koltzenburg, M. pp. 653-667. New York: Elsevier Churchill Livingstone.

Seckinger, P., Klein-Nulend, J., Alander, C., Thompson, R.C., Dayer, J.M. & Raisz, L.G. (1990). Natural and recombinant human IL-1 receptor antagonists block the effects of IL-1 on bone resorption and prostaglandin production. *J Immunol*, **145,** 4181-4.

Seckinger, P., Williamson, K., Balavoine, J.F., Mach, B., Mazzei, G., Shaw, A. & Dayer, J.M. (1987). A urine inhibitor of interleukin 1 activity affects both interleukin 1 alpha and 1 beta but not tumor necrosis factor alpha. *J Immunol*, **139,** 1541-5.

Sehgal, P.B., Helfgott, D.C., Santhanam, U., Tatter, S.B., Clarick, R.H., Ghrayeb, J. & May, L.T. (1988). Regulation of the acute phase and immune responses in viral disease. Enhanced expression of the beta 2-interferon/hepatocyte-stimulating factor/interleukin 6 gene in virus-infected human fibroblasts. *J Exp Med*, **167,** 1951-6.

Serra, J., Campero, M. & Ochoa, J. (1998). Flare and hyperalgesia after intradermal capsaicin injection in human skin. *J Neurophysiol*, **80,** 2801-10.

Shalaby, M.R., Waage, A. & Espevik, T. (1989). Cytokine regulation of interleukin 6 production by human endothelial cells. *Cell Immunol*, **121,** 372-82.

Shenker, N., Haigh, R., Roberts, E., Mapp, P., Harris, N. & Blake, D. (2003). A review of contralateral responses to a unilateral inflammatory lesion. *Rheumatology (Oxford)*, **42,** 1279-86.

Sherrington, C.S. (1906). *The integrative Action of the Nervous System.* New York: Scribner.

Shingu, M., Miyauchi, S., Nagai, Y., Yasutake, C. & Horie, K. (1995). The role of IL-4 and IL-6 in IL-1-dependent cartilage matrix degradation. *Br J Rheumatol*, **34,** 101-6.

Silverman, J.D. & Kruger, L. (1988). Lectin and neuropeptide labeling of separate populations of dorsal root ganglion neurons and associated "nociceptor" thin axons in rat testis and cornea whole-mount preparations. *Somatosens Res*, **5,** 259-67.

Simon, A.K., Seipelt, E. & Sieper, J. (1994). Divergent T-cell cytokine patterns in inflammatory arthritis. *Proc Natl Acad Sci U S A*, **91,** 8562-6.

Simone, D.A., Alreja, M. & LaMotte, R.H. (1991). Psychophysical studies of the itch sensation and itchy skin ("alloknesis") produced by intracutaneous injection of histamine. *Somatosens Mot Res*, **8,** 271-9.

Sims, J.E., Gayle, M.A., Slack, J.L., Alderson, M.R., Bird, T.A., Giri, J.G., Colotta, F., Re, F., Mantovani, A., Shanebeck, K. & et al. (1993). Interleukin 1 signaling occurs exclusively via the type I receptor. *Proc Natl Acad Sci U S A*, **90,** 6155-9.

Sims, J.E., March, C.J., Cosman, D., Widmer, M.B., MacDonald, H.R., McMahan, C.J., Grubin, C.E., Wignall, J.M., Jackson, J.L., Call, S.M. & et al. (1988). cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. *Science*, **241,** 585-9.

Sims, J.E., Painter, S.L. & Gow, I.R. (1995). Genomic organization of the type I and type II IL-1 receptors. *Cytokine*, **7,** 483-90.

Singh, H.N., Blancuzzi, V., Greenwood, S., Skiles, J.W. & O'Byrne, E.M. (1997). Synovial fluid levels of tumor necrosis factor-alpha in the inflamed rat knee: modulation by dexamethasone and inhibitors of matrix metalloproteinase and phosphodiesterase. *Inflamm Res*, **46 Suppl 2,** S153-4.

Skoglund, S. (1956). Anatomical and physiological studies of the knee joint innervation in the cat. *Acta Physiologica Scandinavia*, **124,** 1-101.

Skyba, D.A., Radhakrishnan, R. & Sluka, K.A. (2005). Characterization of a method for measuring primary hyperalgesia of deep somatic tissue. *J Pain*, **6,** 41-7.

Smeets, R.L., Joosten, L.A.B., Arntz, O.J., Bennick, M.B., Takahashi, N., Carlsen, H., Martin, M.U., vsn den Berg, W.B. & van de Loo, A.J. (2005). Soluble Interleukin-1 receptor accessory protein ameliorates collagen-induced arthritis by a different mode of action from that of interleukin-1 receptor antagonist. *Arth & Rheum,* **52,** 2202-2211.

Smeets, T.J., Dolhain, R., Miltenburg, A.M., de Kuiper, R., Breedveld, F.C. & Tak, P.P. (1998). Poor expression of T cell-derived cytokines and activation and proliferation markers in early rheumatoid synovial tissue. *Clin Immunol Immunopathol*, **88,** 84-90.

Smith, G.D., Gunthorpe, M.J., Kelsell, R.E., Hayes, P.D., Reilly, P., Facer, P., Wright, J.E., Jerman, J.C., Walhin, J.P., Ooi, L., Egerton, J., Charles, K.J., Smart, D., Randall, A.D., Anand, P. & Davis, J.B. (2002). TRPV3 is a temperature-sensitive vanilloid receptor-like protein. *Nature*, **418,** 186-90.

Smith-Oliver, T., Noel, L.S., Stimpson, S.S., Yarnall, D.P. & Connolly, K.M. (1993). Elevated levels of TNF in the joints of adjuvant arthritic rats. *Cytokine*, **5,** 298-304.

Smolen, J.S., Redlich, K., Zwerina, J., Aletaha, D., Steiner, G. & Schett, G. (2005). Pro-inflammatory cytokines in rheumatoid arthritis: pathogenetic and therapeutic aspects. *Clin Rev Allergy Immunol*, **28,** 239-48.

Smolen, J.S. & Steiner, G. (2003). Therapeutic strategies for rheumatoid arthritis. *Nat Rev Drug Discov*, **2,** 473-88.

Sokka, T. (2003). Assessment of pain in patients with rheumatic diseases. *Best Practice & Research Clinical Rheumatology, How to Assess Musculoskeletal Conditions*, **17,** 427-449.

Sommer, C. & Kress, M. (2004). Recent findings on how pro-inflammatry cytokines cause pain: pripheral mechanisms in inflammatory and neuropathic hyperalgesia. *Neurosci Lett,* **361,** 184-187.

Song, I-H. & Buttgereit (2006). Non-genomic glucocorticoid effects to provide the basis for new drug developments. *Mol & Cell Endocrin*, **246,** 142-146.

Spencer-Green, G. (2000). Etanercept (Enbrel): update on therapeutic use. *Ann Rheum Dis*, **59 Suppl 1,** i46-9.

Stanfa, L.C. & Dickenson, A.H. (2004). In vivo electrophysiology of dorsal-horn neurons. *Methods Mol Med*, **99,** 139-53.

Steen, K.H. & Reeh, P.W. (1993). Sustained graded pain and hyperalgesia from harmless experimental tissue acidosis in human skin. *Neurosci Lett*, **154,** 113-6.

Steen, K.H., Steen, A.E., Kreysel, H.W. & Reeh, P.W. (1996). Inflammatory mediators potentiate pain induced by experimental tissue acidosis. *Pain*, **66,** 163-70.

Steen, K.H., Steen, A.E. & Reeh, P.W. (1995). A dominant role of acid pH in inflammatory excitation and sensitization of nociceptors in rat skin, in vitro. *J Neurosci*, **15,** 3982-9.

Stein, C., Millan, M.J. & Herz, A. (1988). Unilateral inflammation of the hindpaw in rats as a model of prolonged noxious stimulation: alterations in behavior and nociceptive thresholds. *Pharmacol Biochem Behav*, **31,** 455-51.

Steiner, G., Tohidast-Akrad, M., Witzmann, G., Vesely, M., Studnicka-Benke, A., Gal, A., Kunaver, M., Zenz, P. & Smolen, J.S. (1999). Cytokine production by synovial T cells in rheumatoid arthritis. *Rheumatology (Oxford)*, **38,** 202-13.

Stoerk, H.C., Beielinski, T. & Budzilovich, T. (1954). Chronic polyarthritis in rats injected with spleen in adjuvants. *American journal of pathology*, **30,** 616-321.

Story, G.M., Peier, A.M., Reeve, A.J., Eid, S.R., Mosbacher, J., Hricik, T.R., Earley, T.J., Hergarden, A.C., Andersson, D.A., Hwang, S.W., McIntyre, P., Jegla, T., Bevan, S. & Patapoutian, A. (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*, **112,** 819-29.

Strichartz, G.R., Zhou, Z., Sinnott, C. & Khodorova, A. (2002). Therapeutic concentrations of local anaesthetics unveil the potential role of sodium channels in neuropathic pain. *Novartis Found Symp*, **241,** 189-201; discussion 202-5, 226-32.

Strickland, I.T., Barton, N.J., Brash, H.M., McQueen, D.S., Reeve, A.J., Wilson, A.W. & Chessell, I.P. (2005). A novel behavioural readout for assessing hypersensitivity of knee joints in a murine model of unilateral arthritis. *pA2 online* **3**, 75P

Stuart, J.M., Townes, A.S. & Kang, A.H. (1985). Type II collagen-induced arthritis. *Ann N Y Acad Sci*, **460,** 355-62.

Stucky, C.L. & Lewin, G.R. (1999). Isolectin B(4)-positive and -negative nociceptors are functionally distinct. *J Neurosci*, **19,** 6497-505.

Sugiyama, E., Kuroda, A., Taki, H., Ikemoto, M., Hori, T., Yamashita, N., Maruyama, M. & Kobayashi, M. (1995). Interleukin 10 cooperates with interleukin 4 to suppress inflammatory cytokine production by freshly prepared adherent rheumatoid synovial cells. *J Rheumatol*, **22,** 2020-6.

Sukharev, S.I., Blount, P., Martinac, B. & Kung, C. (1997). Mechanosensitive channels of Escherichia coli: the MscL gene, protein, and activities. *Annu Rev Physiol*, **59,** 633-57.

Suto, K. & Gotoh, H. (1999). Calcium signaling in cold cells studied in cultured dorsal root ganglion neurons. *Neuroscience*, **92,** 1131-5.

Svensson, A., Moller, H., Bjorkner, B., Bruze, M., Leden, I., Theander, J., Ohlsson, K. & Linder, C. (2002). Rheumatoid arthritis, gold therapy, contact allergy and blood cytokines. *BMC Dermataol,* **2.**

Swaak, A.J., van Rooyen, A., Nieuwenhuis, E. & Aarden, L.A. (1988). Interleukin-6 (IL-6) in synovial fluid and serum of patients with rheumatic diseases. *Scand J Rheumatol*, **17,** 469-74.

Swagerty, D.L., Jr. & Hellinger, D. (2001). Radiographic assessment of osteoarthritis. *Am Fam Physician*, **64,** 279-86.

Sweeney, S.E. & Firestein, G.S. (2004). Rheumatoid arthritis: regulation of synovial inflammation. *Int J Biochem Cell Biol*, **36,** 372-8.

Symmons, D., Turner, G., Webb, R., Asten, P., Barrett, E., Lunt, M., Scott, D. & Silman, A. (2002). The prevalence of rheumatoid arthritis in the United Kingdom: new estimates for a new century
10.1093/rheumatology/41.7.793. *Rheumatology*, **41,** 793-800.

Symons, J.A., Eastgate, J.A. & Duff, G.W. (1991). Purification and characterization of a novel soluble receptor for interleukin 1. *J Exp Med*, **174,** 1251-4.

Symons, J.A., Eastgate, J.A. & Duff, G.W. (1990). A soluble binding protein specific for interleukin 1 beta is produced by activated mononuclear cells. *Cytokine*, **2,** 190-8.

Szekanecz, Z., Halloran, M.M., Volin, M.V., Woods, J.M., Strieter, R.M., Kenneth Haines, G., 3rd, Kunkel, S.L., Burdick, M.D. & Koch, A.E. (2000). Temporal expression of inflammatory cytokines and chemokines in rat adjuvant-induced arthritis. *Arthritis Rheum*, **43,** 1266-77.

Szolcsanyi, J. (1996). Capsaicin-sensitive sensory nerve terminals with local and systemic efferent functions: facts and scopes of an unorthodox neuroregulatory mechanism. *Prog Brain Res*, **113,** 343-59.

Szolcsanyi, J., Anton, F., Reeh, P.W. & Handwerker, H.O. (1988). Selective excitation by capsaicin of mechano-heat sensitive nociceptors in rat skin. *Brain Res*, **446,** 262-8.

Taber, R.I. (1973). Predictive value of analgesic assays in mice and rats. *Adv Biochem Psychopharmacol*, **8,** 191-211.

Taetle, R. & Royston, I. (1980). Human T-cell antigens defined by monoclonal antibodies. Absence of T65 on committed myeloid and erythroid progenitors. *Blood*, **56,** 943-6.

Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T. & Kishimoto, T. (1989). Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell*, **58,** 573-81.

Taga, T. & Kishimoto, T. (1997). Gp130 and the interleukin-6 family of cytokines. *Annu Rev Immunol*, **15,** 797-819.

Taiwo, Y.O. & Levine, J.D. (1990). Direct cutaneous hyperalgesia induced by adenosine. *Neuroscience*, **38,** 757-62.

Tak, P.P. (2000). Examination of the synvium and synovial fluid. In *Rheumatoid arthritis: frontiers in pathogenesis and treatment.* eds Firestein, G.S., Panayi, G.S. & Wollheim, F.A. pp. 55-68. NewYork: Oxford University Press.

Takagi, N., Mihara, M., Moriya, Y., Nishimoto, N., Yoshizaki, K., Kishimoto, T., Takeda, Y. & Ohsugi, Y. (1998). Blockage of interleukin-6 receptor ameliorates joint disease in murine collagen-induced arthritis. *Arthritis Rheum*, **41,** 2117-21.

Tarner, I.H., Nakajima, A., Seroogy, C.M., Ermann, J., Levicnik, A., Contag, C.H. & Fathman, C.G. (2002). Retroviral gene therapy of collagen-induced arthritis by local delivery of IL-4. *Clin Immunol*, **105,** 304-14.

Tavernarakis, N. & Driscoll, M. (1997). Molecular modeling of mechanotransduction in the nematode Caenorhabditis elegans. *Annu Rev Physiol*, **59,** 659-89.

Taylor, P. & Feldmann, M. (2004). Rheumatoid arthritis: pathogenic mechanisms and therapeutic targets. *Drug Discovery Today: Disease Mechanisms*, **1,** 289-295.

te Velde, A.A., Huijbens, R.J., Heije, K., de Vries, J.E. & Figdor, C.G. (1990). Interleukin-4 (IL-4) inhibits secretion of IL-1 beta, tumor necrosis factor alpha, and IL-6 by human monocytes. *Blood*, **76,** 1392-7.

Tetlow, L.C. & Woolley, D.E. (1995). Distribution, activation and tryptase/chymase phenotype of mast cells in the rheumatoid lesion. *Ann Rheum Dis*, **54,** 549-55.

Thornton, S., Duwel, L.E., Boivin, G.P., Ma, Y. & Hirsch, R. (1999). Association of the course of collagen-induced arthritis with distinct patterns of cytokine and chemokine messenger RNA expression. *Arthritis Rheum*, **42,** 1109-18.

Thresh, L.T. (1946). Isolation of Capsaicin. *Pharm. J.*, **6,** 941.

Tobin, D., Madsen, D., Kahn-Kirby, A., Peckol, E., Moulder, G., Barstead, R., Maricq, A.& Bargmann, C. (2002). Combinatorial expression of TRPV channel proteins defines their sensory functions and subcellular localization in C.elegans neurons. *Neuron,* **35,** 307-18.

Todd, A.J. & Koerber, R. (2006). Neuroanatmonical substrates of spinal nociception. In *Textbook of Pain.* ed Wall, P.D. pp. 1-16. New York: Churchill Livingstone.

Tohda, C., Sasaki, M., Konemura, T., Sasamura, T., Itoh, M. & Kuraishi, Y. (2001). Axonal transport of VR1 capsaicin receptor mRNA in primary afferents and its participation in inflammation-induced increase in capsaicin sensitivity. *J Neurochem*, **76,** 1628-35.

Tominaga, M., Caterina, M.J., Malmberg, A.B., Rosen, T.A., Gilbert, H., Skinner, K., Raumann, B.E., Basbaum, A.I. & Julius, D. (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron*, **21,** 531-43.

Tonussi, C.R. & Ferreira, S.H. (1992). Rat knee-joint carrageenin incapacitation test: an objective screen for central and peripheral analgesics. *Pain*, **48,** 421-7.

Tonussi, C.R. & Ferreira, S.H. (1999). Tumour necrosis factor-alpha mediates carrageenin-induced knee-joint incapacitation and also triggers overt nociception in previously inflamed rat knee-joints. *Pain*, **82,** 81-7.

Torebjork, H.E. & Hallin, R.G. (1974). Identification of afferent C units in intact human skin nerves. *Brain Res*, **67,** 387-403.

Torebjork, H.E., Lundberg, L.E. & LaMotte, R.H. (1992). Central changes in processing of mechanoreceptive input in capsaicin-induced secondary hyperalgesia in humans. *J Physiol*, **448,** 765-80.

Tracey, K.J. & Cerami, A. (1994). Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu Rev Med*, **45,** 491-503.

Trang, L.E., Granstrom, E. & Lovgren, O. (1977). Levels of prostaglandins F2 alpha and E2 and thromboxane B2 in joint fluid in rheumatoid arthritis. *Scand J Rheumatol*, **6,** 151-4.

Treede, R.D., Rolke, R., Andrews, K. & Magerl, W. (2002). Pain elicited by blunt pressure: neurobiological basis and clinical relevance. *Pain*, **98,** 235-40.

Tsuboi, M., Kawakami, A., Nakashima, T., Matsuoka, N., Urayama, S., Kawabe, Y., Fujiyama, K., Kiriyama, T., Aoyagi, T., Maeda, K. & Eguchi, K. (1999). Tumor necrosis factor-alpha and interleukin-1beta increase the Fas-mediated apoptosis of human osteoblasts. *J Lab Clin Med*, **134,** 222-31.

Turner, M., Chantry, D., Buchan, G., Barrett, K. & Feldmann, M. (1989). Regulation of expression of human IL-1 alpha and IL-1 beta genes. *J Immunol*, **143,** 3556-61.

Uhlig, T., Hagen, K.B. & Kvien, T.K. (2002). Why do patients with chronic musculoskeletal disorders consult their primary care physicians? *Curr Opin Rheumatol*, **14,** 104-8.

Ulfgren, A.K., Grondal, L., Lindblad, S., Khademi, M., Johnell, O., Klareskog, L. & Andersson, U. (2000). Interindividual and intra-articular variation of proinflammatory cytokines in patients with rheumatoid arthritis: potential implications for treatment. *Ann Rheum Dis*, **59,** 439-47.

Ulfgren, A.K., Lindblad, S., Klareskog, L., Andersson, J. & Andersson, U. (1995). Detection of cytokine producing cells in the synovial membrane from patients with rheumatoid arthritis. *Ann Rheum Dis*, **54,** 654-61.

Urch, C.E. & Dickenson, A.H. (2003). In vivo single unit extracellular recordings from spinal cord neurones of rats. *Brain Res Brain Res Protoc*, **12,** 26-34.

Vale, M.L., Benevides, V.M., Sachs, D., Brito, G.A., da Rocha, F.A., Poole, S., Ferreira, S.H., Cunha, F.Q. & Ribeiro, R.A. (2004). Antihyperalgesic effect of pentoxifylline on experimental inflammatory pain. *Br J Pharmacol*, **143,** 833-44.

Van Damme, J., Cayphas, S., Opdenakker, G., Billiau, A. & Van Snick, J. (1987). Interleukin 1 and poly(rI).poly(rC) induce production of a hybridoma growth factor by human fibroblasts. *Eur J Immunol*, **17,** 1-7.

van de Loo, A.A., Arntz, O.J., Bakker, A.C., van Lent, P.L., Jacobs, M.J. & van den Berg, W.B. (1995). Role of interleukin 1 in antigen-induced exacerbations of murine arthritis. *Am J Pathol*, **146,** 239-49.

van de Loo, F.A., Arntz, O.J., Otterness, I.G. & van den Berg, W.B. (1992). Protection against cartilage proteoglycan synthesis inhibition by antiinterleukin 1 antibodies in experimental arthritis. *J Rheumatol*, **19,** 348-56.

van de Loo, F.A., Kuiper, S., van Enckevort, F.H., Arntz, O.J. & van den Berg, W.B. (1997). Interleukin-6 reduces cartilage destruction during experimental arthritis. A study in interleukin-6-deficient mice. *Am J Pathol*, **151,** 177-91.

van den Berg, W.B. (2001). Anti-cytokine therapy in chronic destructive arthritis. *Arthritis Res*, **3,** 18-26.

van den Berg, W.B. (1999). The role of cytokines and growth factors in cartilage destruction in osteoarthritis and rheumatoid arthritis. *Z Rheumatol*, **58,** 136-41.

van den Bosch, F. (1998). eHu IL-4 in subjects with active rheumatoid arthritis (RA): a phase I dose escalating safety study. *Arthritis & Rheumatism*, **41,** S56.

van Eden, W., Thole, J.E., van der Zee, R., Noordzij, A., van Embden, J.D., Hensen, E.J. & Cohen, I.R. (1988). Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature*, **331,** 171-3.

Van Hees, J. & Gybels, J.M. (1972). Pain related to single afferent C fibers from human skin. *Brain Res*, **48,** 397-400.

Van Lent, P.L., Van De Loo, F.A., Holthuysen, A.E., Van Den Bersselaar, L.A., Vermeer, H. & Van Den Berg, W.B. (1995). Major role for interleukin 1 but not for tumor necrosis factor in early cartilage damage in immune complex arthritis in mice. *J Rheumatol*, **22,** 2250-8.

van Roon, J.A., Bijlsma, J.W. & Lafeber, F.P. (2002). Suppression of inflammation and joint destruction in rheumatoid arthritis may require a concerted action of Th2 cytokines. *Curr Opin Investig Drugs*, **3,** 1011-6.

van Roon, J.A., van Roy, J.L., Gmelig-Meyling, F.H., Lafeber, F.P. & Bijlsma, J.W. (1996). Prevention and reversal of cartilage degradation in rheumatoid arthritis by interleukin-10 and interleukin-4. *Arthritis Rheum*, **39,** 829-35.

Van Snick, J. (1990). Interleukin-6: an overview. *Annu Rev Immunol*, **8,** 253-78.

Vannier, E., de Waal Malefyt, R., Salazar-Montes, A., de Vries, J.E. & Dinarello, C.A. (1996). Interleukin-13 (IL-13) induces IL-1 receptor antagonist gene expression and protein synthesis in peripheral blood mononuclear cells: inhibition by an IL-4 mutant protein. *Blood*, **87,** 3307-15.

Vannier, E., Miller, L.C. & Dinarello, C.A. (1992). Coordinated antiinflammatory effects of interleukin 4: interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and synthesis of interleukin 1 receptor antagonist. *Proc Natl Acad Sci U S A*, **89,** 4076-80.

Veys, E.M., Menkes, C.J. & Emery, P. (1997). A randomized, double-blind study comparing twenty-four-week treatment with recombinant interferon-gamma versus placebo in the treatment of rheumatoid arthritis. *Arthritis Rheum*, **40,** 62-8.

Vigers, G.P., Caffes, P., Evans, R.J., Thompson, R.C., Eisenberg, S.P. & Brandhuber, B.J. (1994). X-ray structure of interleukin-1 receptor antagonist at 2.0-A resolution. *J Biol Chem*, **269,** 12874-9.

von Frey, M. (1894). Beitrag zur physiologie des schmerzsinns. *Koenigl. Saechs. Ges. Wiss. , Math-Phys*, **46,** 184-186.

von Frey, M. (1922). Versuche uber schmerzerregende reize. *Z.Biol*, **76,** 1-24.

Wagner, S., Fritz, P., Einsele, H., Sell, S. & Saal, J.G. (1997). Evaluation of synovial cytokine patterns in rheumatoid arthritis and osteoarthritis by quantitative reverse transcription polymerase chain reaction. *Rheumatol Int*, **16,** 191-6.

Walker, J.M. & Huang, S.M. (2002). Endocannabinoids in pain modulation. *Prostaglandins Leukot Essent Fatty Acids*, **66,** 235-42.

Walker, K.M., Urban, L., Medhurst, S.J., Patel, S., Panesar, M., Fox, A.J. & McIntyre, P. (2003). The VR1 antagonist capsazepine reverses mechanical hyperalgesia in models of inflammatory and neuropathic pain. *J Pharmacol Exp Ther*, **304,** 56-62.

Walker, R.G., Willingham, A.T. & Zucker, C.S. (2000). A drosophilia mechanosensory transduction channel. *Science,* **287,** 2132-3

Wall, P.D. (1984). Introduction. In *Textbook of Pain.* ed Wall, P.D. pp. 1-16. New York: Churchill Livingstone.

Wang, Y., Huang, C., Cao, Y. & Han, J.S. (2000). Repeated administration of low dose ketamine for the treatment of monoarthritic pain in the rat. *Life Sci*, **67,** 261-7.

Ware, J.E., Jr. & Sherbourne, C.D. (1992). The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. *Med Care*, **30,** 473-83.

Weinblatt, M.E. (1999). rHuIL-10 (tenovil) plus methotrexate (MTX) in active rheumaoid arthritis: A phase I/II study. *Arthritis & Rheumatism*, **42,** S170.

Weissenbach, J., Chernajovsky, Y., Zeevi, M., Shulman, L., Soreq, H., Nir, U., Wallach, D., Perricaudet, M., Tiollais, P. & Revel, M. (1980). Two interferon mRNAs in human fibroblasts: in vitro translation and Escherichia coli cloning studies. *Proc Natl Acad Sci U S A*, **77,** 7152-6.

Weitz, Z., Moak, S.A. & Greenwald, R.A. (1988). Degradation of hyaluronic acid by neutrophil derived oxygen radicals is stimulus dependent. *J Rheumatol*, **15,** 1250-3.

Wemmie, J.A., Price, M.P. & Welsh, M.J. (2006). Acid-sensing ion channels: advances, questions and therapeutic opportunities. *Trends in neurosci*, **29,** 578-86.

Wendling, D., Racadot, E. & Wijdenes, J. (1993). Treatment of severe rheumatoid arthritis by anti-interleukin 6 monoclonal antibody. *J Rheumatol*, **20,** 259-62.

Wesche, H., Korherr, C., Kracht, M., Falk, W., Resch, K. & Martin, M.U. (1997). The interleukin-1 receptor accessory protein (IL-1RAcP) is essential for IL-1-induced activation of interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinases (SAP kinases). *J Biol Chem*, **272,** 7727-31.

Westman, M., Korotkova, M., af Klint, E., Stark, A., Audoly, L.P., Klareskog, L., Ulfgren, A.K. & Jakobsson, P.J. (2004). Expression of microsomal prostaglandin E synthase 1 in rheumatoid arthritis synovium. *Arthritis Rheum*, **50,** 1774-80.

Wilder, R.L. (1988). Streptococcal cell-wall-induced arthritis in rats: an overview. *Tissue React*, **10,** 1-5.

Williams, R.O., Feldmann, M. & Maini, R.N. (1992). Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc Natl Acad Sci U S A*, **89,** 9784-8.

Willis, W.D. and Coggeshall, R.E. (1991). Sensory mechanisms of the spinal cord. New York: Plenium Press.

Wilson, A.W., Medhurst, S.J., Dixon, C.I., Bontoft, N.C., Winyard, L.A., Brackenborough, K.T., Alba, J.D., Clarke, C.J., Gunthorpe, M.J. & Hicks, G.A. (2006). An animal model of chronic inflammatory pain: Pharmacological and temporal differentiation from acute models. *European Journal of Pain*, **10,** 537-549.

Wixson, S.K., White, W.J., Hughes, H.C., Jr., Lang, C.M. & Marshall, W.K. (1987). The effects of pentobarbital, fentanyl-droperidol, ketamine-xylazine and ketamine-diazepam on arterial blood pH, blood gases, mean arterial blood pressure and heart rate in adult male rats. *Lab Anim Sci*, **37,** 736-42.

Wollheim, F.A. (2001). Advances in Targeted Therapies III, Nassau, Bahamas, 27 April-1 May 2001. *Arthritis Res*, **3,** E0005.

Woo, Y.C., Park, S.S., Subieta, A.R. & Brennan, T.J. (2004). Changes in tissue pH and temperature after incision indicate acidosis may contribute to postoperative pain. *Anesthesiology*, **101,** 468-75.

Wood, D.D., Ihrie, E.J., Dinarello, C.A. & Cohen, P.L. (1983). Isolation of an interleukin-1-like factor from human joint effusions. *Arthritis Rheum*, **26,** 975-83.

Woodbury, J.W. & Patton, H.D. (1952). Electrical activity of single spinal cord elements. *Cold Spring Harb Symp Quant Biol*, **17,** 185-8.

Wooley, P.H. (2004). The usefulness and the limitations of animal models in identifying targets for therapy in arthritis. *Best Pract Res Clin Rheumatol*, **18,** 47-58.

Wooley, P., Whalen, J.D., Chapman, D.L., Berger, A.E., Richard, K.A., Aspar, D.G. & Staite, N.D. (1993a). The effect of an interleukin-1 receptor antagonist protein on type II collagen-indced arthritis and antigen-induced arthritis in mice. *Arthritis & Rheumatism*, **36,** 1305-1314.

Wooley, P.H., Dutcher, J., Widmer, M.B. & Gillis, S. (1993b). Influence of a recombinant human soluble tumor necrosis factor receptor FC fusion protein on type II collagen-induced arthritis in mice. *J Immunol*, **151,** 6602-7.

Wu, G., Whiteside, G.T., Lee, G., Nolan, S., Niosi, M., Pearson, M.S. & Ilyin, V.I. (2004). A-317491, a selective P2X3/P2X2/3 receptor antagonist, reverses inflammatory mechanical hyperalgesia through action at peripheral receptors in rats. *Eur J Pharmacol*, **504,** 45-53.

Xu, H., Ramsey, I.S., Kotecha, S.A., Moran, M.M., Chong, J.A., Lawson, D., Ge, P., Lilly, J., Silos-Santiago, I., Xie, Y., DiStefano, P.S., Curtis, R. & Clapham, D.E. (2002). TRPV3 is a calcium-permeable temperature-sensitive cation channel. *Nature*, **418,** 181-6.

Xu, X.J., Hao, J.X., Andell-Jonsson, S., Poli, V., Bartfai, T. & Wiesenfeld-Hallin, Z. (1997). Nociceptive responses in interleukin-6-deficient mice to peripheral inflammation and peripheral nerve section. *Cytokine*, **9,** 1028-33.

Yasukawa, K., Hirano, T., Watanabe, Y., Muratani, K., Matsuda, T., Nakai, S. & Kishimoto, T. (1987). Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene. *Embo J*, **6,** 2939-45.

Ye, K., Koch, K.C., Clark, B.D. & Dinarello, C.A. (1992). Interleukin-1 down-regulates gene and surface expression of interleukin-1 receptor type I by destabilizing its mRNA whereas interleukin-2 increases its expression. *Immunology*, **75,** 427-34.

Yu, Y.C., Koo, S.T., Kim, C.H., Lyu, Y., Grady, J.J. & Chung, J.M. (2002). Two variables that can be used as pain indices in experimental animal models of arthritis. *J Neurosci Methods*, **115,** 107-13.

Zemlan, F.P., Behbehani, M.M. & Murphy, R.M. (1988). Serotonin receptor subtypes and the modulation of pain transmission. *Prog Brain Res*, **77,** 349-55.

Zhang, F.-C., Hou, Y., Huang, F., Wu, D.-H., Bao, C.-D., Ni, L.-Q. & Yao, C. (2006). Infliximab versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a preliminary study from China. *APLAR Journal of Rheumatology*, **9,** 127-130.

Zhang, Y., Lin, J.X. & Vilcek, J. (1988). Synthesis of interleukin 6 (interferon-beta 2/B cell stimulatory factor 2) in human fibroblasts is triggered by an increase in intracellular cyclic AMP. *J Biol Chem*, **263,** 6177-82.

Zhong, J., Dietzel, I.D., Wahle, P., Kopf, M. & Heumann, R. (1999). Sensory impairments and delayed regeneration of sensory axons in interleukin-6-deficient mice. *J Neurosci*, **19,** 4305-13.

Zhong, J. & Heumann, R. (1995). Lesion-induced interleukin-6 mRNA expression in rat sciatic nerve. *Ann N Y Acad Sci*, **762,** 488-90.

Zhou, S., Bonasera, L. & Carlton, S.M. (1996). Peripheral administration of NMDA, AMPA or KA results in pain behaviors in rats. *Neuroreport*, **7,** 895-900.

Zotterman, Y. (1933). Studies in the peripheral nervous system mechanism of pain. *Acta medica Scandinavica*, **80**.

Zucali, J.R., Dinarello, C.A., Oblon, D.J., Gross, M.A., Anderson, L. & Weiner, R.S. (1986). Interleukin 1 stimulates fibroblasts to produce granulocyte-macrophage colony-stimulating activity and prostaglandin E2. *J Clin Invest*, **77,** 1857-63.

Zwerina, J., Hayer, S., Tohidast-Akrad, M., Bergmeister, H., Redlich, K., Feige, U., Dunstan, C., Kollias, G., Steiner, G., Smolen, J. & Schett, G. (2004). Single and combined inhibition of tumor necrosis factor, interleukin-1, and RANKL pathways in tumor necrosis factor-induced arthritis: effects on synovial inflammation, bone erosion, and cartilage destruction. *Arthritis Rheum*, **50,** 277-90.

Zwerina, J., Redlich, K., Schett, G. & Smolen, J.S. (2005). Pathogenesis of rheumatoid arthritis: targeting cytokines. *Ann N Y Acad Sci*, **1051,** 716-29.

Zwick, M., Davis, B.M., Woodbury, C.J., Burkett, J.N., Koerber, H.R., Simpson, J.F. & Albers, K.M. (2002). Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. *J Neurosci*, **22,** 4057-65.

Zygmunt, P.M., Petersson, J., Andersson, D.A., Chuang, H., Sorgard, M., Di Marzo, V., Julius, D. & Hogestatt, E.D. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature*, **400,** 452-7.

Appendix 1 - Drugs used in this study

Generic Name	Chemical Name	Formula Weight	Supplier	Solvent
Celecoxib	4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide	381.38	GlaxoSmithKline	Ethanol 5% PEG 45% dH_20 50%
IL1β	Interleukin 1β	~ 17 kD	Bioclone, USA	Saline
IL6	Interleukin 6	~22 kDa	Bioclone, USA	Saline
Morphine	Morphine sulfate salt pentahydrate	758.83	Sigma	Saline
Prednisolone	Pregna- 1,4- diene-3,20-dione, 11,17,21-trihydroxy-,(11ß)	360.44	Sigma	Ethanol 5% PEG 45% dH_20 50%

Solutions

Saline: 0.9 g NaCl in 100 ml dH_20

10% Formalin: 10 ml 40% formaldehyde (w v^{-1})
0.9 g NaCl
90 ml hH_20

3.15% sodium citrate: 3.15 g sodium citrate in 1000 ml sterile saline

Appendix II – Publications

Mice lacking the TRPV1 receptor develop milder joint inflammation following FCA

Barton, N.J.[1], McQueen,D.S.[1], Gauldie,S.D.[1], Wilson,A.W.[2], Clayton,N.M.[2], Chessel,I.P.[2]
[1]Division of Neuroscience, University of Edinburgh, 1 George Sq, EH8 9JZ, UK,
[2]Neurology CEDD, GlaxoSmithKline R&D Ltd, Harlow, Essex CM19 5AW, UK

The TRPV1 receptor (TRPV1R) is a non-selective ligand-gated cation channel expressed predominantly by nociceptive sensory neurons (Caterina *et al.*, 1997). The aim of this study was to determine the role of this receptor in the development and maintenance of joint inflammation evoked by Freunds Complete Adjuvant (FCA) in a murine model of chronic unilateral arthritis. The body weights, joint diameters and weight distribution on hind limbs (Clayton *et al.*, 1997) were measured in male C57BL6 wild type (WT) and TRPV1R-/- mice (Davis *et al.* 2000). Five C57BL6 WT and 10 TRPV1R-/- mice were injected with FCA (200µg/20µl intra articular) in the left stifle joint under 3% halothane anaesthesia, as described by Gauldie *et al.* (2004). Four C57BL6 WT and 10 TRPV1R-/- mice were injected with vehicle (20 µl heavy liquid paraffin). Measurements were made three times per week for 30d. Intra-articular FCA caused an increase in ipsilateral joint diameter in WT mice that was significantly greater than that observed in TRPV1R-/- mice ($P<0.01$; MannWhitney U), see Figure 1. Weight bearing on the hind limbs shifted from equal weight distribution on both joints towards the untreated joint after FCA injection. The difference between the two joints was statistically significant in both groups ($P<0.05$; Mann Whitney U). There were no significant differences between the strains ($P>0.05$; Mann Whitney U; Fig.1).

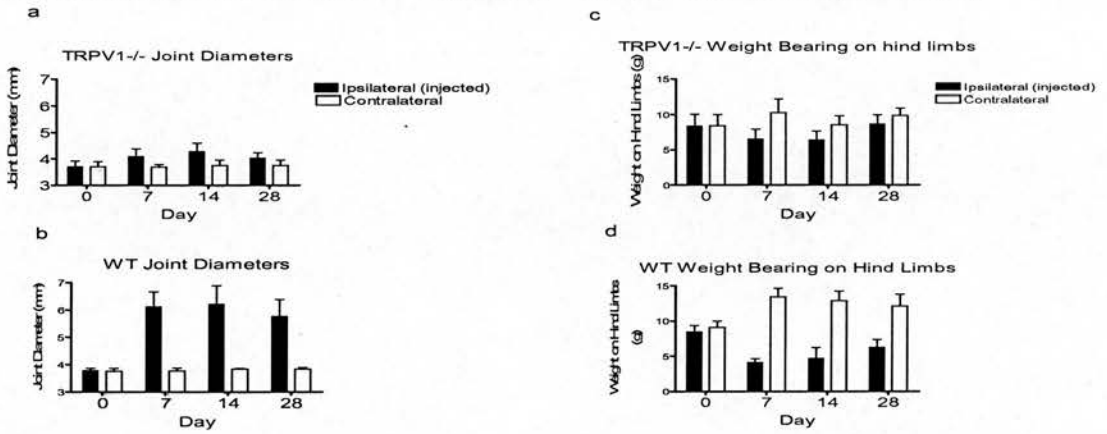


Figure 1. Ipsilateral (injected with FCA) and contralateral knee diameters of male (a) TRPV1-/-mice and (b) WT mice. Weight bearing of male (c) TRPV1-/- mice and (d) WT mice.

Our results indicate that TRPV1Rs play a key role in the development of inflammation, in agreement with Keeble *et al.* (2004). In addition, data from weight distribution on hind limbs suggest that the TRPV1Rs play a role in hyperalgesia following i.art FCA, however the difference from WT was not statistically significant, probably due to the variability of the data. In conclusion our data suggests TRPV1R is involved in joint inflammation and hyperalgesia. However, since neither were completely attenuated in the knock out mice, it is probable that multiple mediators, receptors and mechanisms are involved in the development and maintenance of inflammatory joint disease.

Caterina, M.J. *et al.*(1997). *Nature,* **389**, 816-24.
Clayton, N.M. *et al.* (1997) *Br J Pharmacol* **120**, 75P
Davis, J.B. *et al.* (2000). Nature, **405,** 183-187
Gauldie S.D. *et al.* (2004). *J Neurosci Methods*, **139,** 281-291
Keeble, J.E. *et al.* (2004). *Proc British Pharm Soc, pA2 online,* **21,** 43P

MICE LACKING THE TRPV1 RECEPTOR DEVELOP A MILDER JOINT INFLAMMATION FOLLOWING FCA

Barton, N.J.[1], McQueen,D.S.[1], Gauldie,S.D.[1], Wilson,A.W.[2], Clayton,N.M.[2], Chessel,I.P.[2]
[1]Division of Neuroscience, University of Edinburgh, Medical College, 1 George Sq, EH8 9JZ, UK,
[2]Neurology CEDD, GlaxoSmithKline R&D Ltd, Harlow, Essex CM19 5AW, UK

1.Introduction

The TRPV1 receptor (TRPV1R) is a non-selective ligand-gated cation channel expressed predominantly by nociceptive sensory neurons (Caterina *et al.*, 1997).

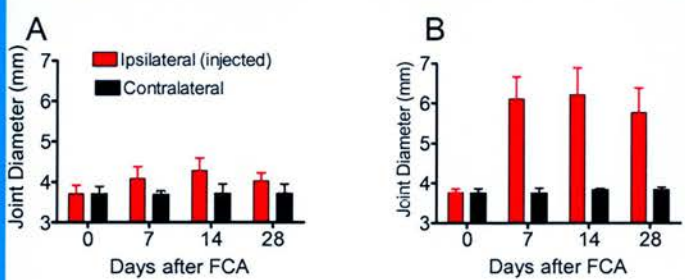
Aim – Experiments were undertaken to test the hypothesis that the TRPV1 receptor plays a key role in the development and maintenance of joint inflammation in mice. Inflammation of the stifle (knee) joint was evoked by Freund's Complete Adjuvant (FCA) in TRPV1R -/- mice (Davis *et al*, 2000).

2.Methods

The body weights, joint diameters (measured using digital microcallipers), and weight distribution between hind limbs – an index of hyperalgesia (Clayton *et al*., 1997) -were measured in male C57BL6 wild type (WT) and TRPV1R-/- mice (15-25g). Five C57BL6 WT and 10 TRPV1R-/- mice were injected with FCA (200µg/20µl intra articular, i.art) in the left stifle joint under 3% halothane anaesthesia, as described by Gauldie *et al.* (2004). Four C57BL6 WT and 10 TRPV1R-/- mice were injected with i.art vehicle (20 µl heavy liquid paraffin). Measurements were made three times per week for 30 days.

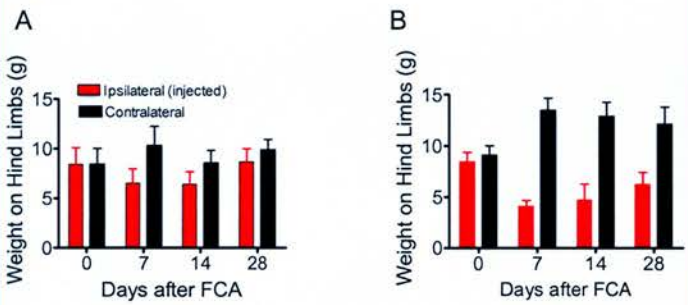
3.Results

Figure 1.



In TRPV1-/- (Fig 1A) and C57B6 WT (Fig 1B) mice there was a significant increase in knee diameter of the injected joint in comparison with the contralateral limb ($P<0.05$ for both WT and TRPV1-/- vs. HLP, Mann Whitney test). The difference between the WT and the TRPV1-/- injected joints was statistically significant ($P<0.001$, Mann-Whitney). This finding is in agreement with Keeble *et al* (2004).

Figure 2



Weight distribution on hind limbs of TRPV1-/- (Fig 2A) and C57B6 WT (Fig 2B) mice. Weight bearing shifted from equal distribution on both limbs before FCA towards the untreated joint after i.art FCA. The difference between the ipsilateral and the contralateral joints was significant in both groups ($P<0.05$, Mann Whitney test). There was a significant difference between the WT and the TRPV1-/- ipsilateral joints on day 7, 14 and 28.

Keeble *et al* (2004) found plasma extravasation from joints after FCA i.art was reduced in TRPV1-/- in comparison with WT mice, which is consistent with our data on joint swelling .

4.Summary

- **TRPV1R -/- mice develop a milder joint inflammation following i.art FCA, compared with WT mice, as measured by joint diameter over 30 days.**
- **Data from weight distribution on hind limbs suggest that the TRPV1R also plays a role in hyperalgesia following i.art FCA, however this is not statistically significant, probably due to the small sample size and variability of the data.**
- **Our data suggests a role for TRPV1R in joint inflammation and hyperalgesia. Other mediators and receptors also appear to be involved in hyperalgesia and allodynia.**

References

Caterina, M.J. *et al.*(1997). *Nature,* **389**, 816-24.
Clayton, N.M. *et al.* (1997) *Br J Pharmacol (Proc Suppl.)* **75P**
Davis, J.B. *et al.* (2000). Nature, **405,** 183-187
Gauldie S.D. *et al.* (2004). *J Neurosci Methods*, **139,** 281-291
Keeble, J.E. *et al.* (2004). *Proc British Pharm Soc, pA2 online,* **21,** 43P

THE RELEVANCE OF THE CHILLI PEPPER RECEPTOR IN ARTHRITIS

N. Barton[1], D.S.McQueen[1], D.Thomson[1], A.W.Wilson[2]. I.P.Chessell[2]
[1]Division of Neuroscience, University of Edinburgh, EH8 9JZ, UK.
[2]Neurology CEDD, GlaxoSmithKline R&D Ltd, Harlow CM19 5AW, UK.

Chilli peppers contain capsaicin, a chemical which interacts with a protein called the capsaicin or TRPV1 receptor on nerve endings, causing the hot burning sensation associated with eating spicy foods. Capsaicin also selectively activates a group of sensory nerves responsible for detecting painful stimuli; TRPV1 receptor expression increase during autoimmune inflammatory disease, such as rheumatoid arthritis. We are using genetic engineering techniques to study the role of TRPV1 receptors in a murine model of chronic arthritis. Mild joint inflammation was induced under general anaesthesia with Freund's Complete Adjuvant in TRPV1 -/- (genetically manipulated to "knockout" the TRPV1 receptor) and WT (normal) mice. Functional measures of joint inflammation were made in 20 TRPV1-/- and 9 WT mice. Joint swelling and hypersensitivity were significantly reduced in the knockout animals compared with the WT. These results indicate that TRPV1 receptors play a key role in the development of joint inflammation, contributing to the hypersensitivity of arthritis. However, as neither swelling nor hypersensitivity were completely eliminated in the knock out mice, other mediators must also be involved in the development and maintenance of arthritis. The study shows that the TRPV1 receptor that is activated by capsaicin in Chilli's is important in chronic joint pain. Therefore this receptor, together with its as yet undiscovered endogenous ligand (the substance produced by the body which activates the TRPV1 receptor), are important targets for developing novel anti-inflammatory or analgesic drugs to relieve the symptoms of arthritis.

The Role of the Chilli Pepper Receptor in Arthritis

N.J.Barton[1], D.S.McQueen[1], D.Thomson[1], A.W.Wilson[2], I.P.Chessell[2]
[1]Division of Neuroscience, University of Edinburgh, Medical College, 1 George Sq, EH8 9JZ, UK,
[2]Neurology CEDD, GlaxoSmithKline R&D Ltd, Harlow, Essex CM19 5AW, UK

Introduction

Chilli Peppers contain capsaicin, a chemical which interacts with TRPV1 receptors on nerve endings, causing the burning sensation experienced when eating spicy foods. The same receptor also plays an important role in the modulation of the way we feel pain. TRPV1 receptors are located on 'nociceptors' which are specialised neurones which relay noxious, painful sensations from the periphery to the spinal cord and then into the brain. The expression of TRPV1 receptors increases during autoimmune inflammatory diseases, including rheumatoid arthritis. It is therefore likely that this receptor and its mysterious endogenous ligand are valuable targets for anti-inflammatory or analgesic drugs.

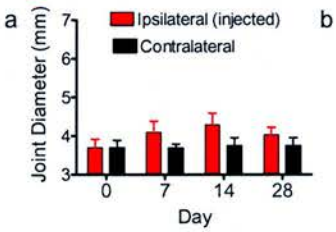
Aim

Use a genetically engineered mouse lacking the TRPV1 receptor (Davis *et al* 2000) to determine the role of TRPV1R in the development and maintenance of inflammation and hypersensitivity in a murine model of rheumatoid arthritis.

Methods

The body weights, joint diameters (measured using digital micro callipers), and weight distribution between hind limbs (an index of hypersensitivity; Clayton *et al* 1997) were measured in male C57BL6 wild type (WT; normal) and TRPV1R-/- mice (15-25g). Five C57BL6 WT and 10 TRPV1R-/- mice were injected with Freunds Complete Adjuvant (FCA; 200µg in a volume of 20µl intra articular, i.art) in the left stifle (knee) joint under 3% halothane anaesthesia, as described by Gauldie *et al* 2004. Four C57BL6 WT and 10 TRPV1R-/- mice were injected with vehicle (20 µl heavy liquid paraffin; i.art). Measurements were made three times per week for 30 days. At the end of the study, both ipsilateral and contralateral joints were removed, fixed in formalin and processed for histological staining. Haematoxylin-eosin staining was carried out to study general signs of joint inflammation.

Results



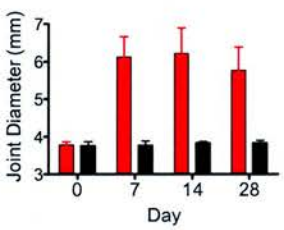


Fig 1. In TRPV1-/- (a) and C57B6 WT (b) mice there was a significant increase in knee diameter of the injected joint in comparison with the contralateral limb ($P<0.05$ for both WT and TRPV1-/- vs. HLP, Mann Whitney test). The difference between the WT and the TRPV1-/- injected joints was statistically significant ($P<0.001$, Mann-Whitney). The inflammation was almost completely abolished in TRPV1-/- mice.

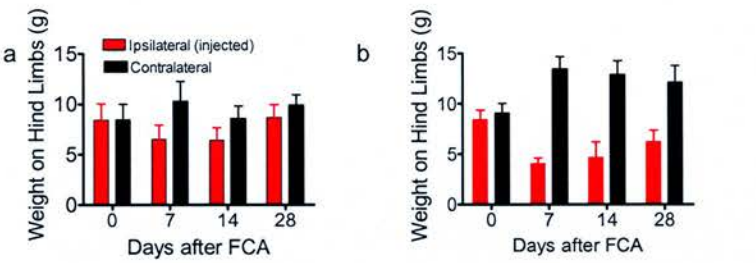


Fig 2. Weight distribution on hind limbs of TRPV1-/- (a) and C57B6 WT (b) mice. Weight bearing shifted from equal distribution on both limbs before FCA towards the untreated joint after i.art FCA. The difference between the ipsilateral and the contralateral joints was significant in both groups ($P<0.05$, Mann Whitney test). There was a significant difference between the WT and the TRPV1-/- ipsilateral joints on days 7, 14 and 28.

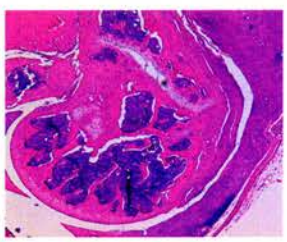


Fig 3. Haematoxylin-eosin staining shows a reduced number of inflammatory cells in the inflamed joint of the TRPV1-/- joint in comparison with the response evoked in WT mice.

Summary

This model of arthritis mimics the pathology of human disease and is important for developing new anti-inflammatory agents to relieve the chronic pain of arthritic disease.

We have shown that both joint inflammation and hypersensitivity were reduced in the knockout animals, suggesting TRPV1 is crucial in their development in chronic joint pain. However, as neither were completely attenuated other mechanisms must also play a role in the development and maintenance of arthritis.

Drugs targeting the Chilli Pepper receptor have the potential to be peripherally acting anti-inflammatory analgesics. would therefore be anti-ammatory and analgesic in action.

Acknowledgements

With thanks to GSK for supplying TRPV1 k/o mice and funding my PhD research.

References

Caterina, M.J. *et al.*(1997). *Nature*, **389**, 816-24.
Clayton, N.M. *et al.* (1997) *Br J Pharmacol (Proc Suppl.)* **75P**
Davis, J.B. *et al.* (2000). Nature, **405**, 183-187
Gauldie S.D. *et al.* (2004). *J Neurosci Methods*, **139**, 281-291

Intra-articular IL1β or IL6 induces hypersensitivity and allodynia in the rat knee joint

N.J.Barton[1], I.T.Strickland[1], A.J.Reeve[2], D.S.McQueen[1], I.P. Chessell[2]

[1]Division of Neuroscience, University of Edinburgh, 1 George Sq, EH8 9JZ, UK

[2]Neurology CEDD, GlaxoSmithKline R&D Ltd, Harlow, Essex CM19 5AW, UK

IL1β and IL6 are pro-inflammatory cytokines present in high levels in rheumatoid arthritic joints; their blood concentrations correlate with disease severity (Eastgate *et al.* 1988; Houssiau *et al.*1988). The aim of this experiment was to determine if exogenous intra-articular IL1β or IL6 resulted in hypersensitivity or allodynia in the rat. Body weights, knee joint diameters, weight distribution between hind limbs (Clayton *et al.*, 1997) and the withdrawal threshold to a discrete pressure applied to the knee joint (PAD; Strickland *et al.* 2005), a measure of allodynia, were measured in 42 male Wistar rats (150-175g). Animals were divided into 7 groups, 1, 3 and 10µg IL1β or IL6 (rat recombinant; Bioclone Inc, USA) and vehicle. Rats were transiently anaesthetised with 3% halothane in O_2 and a 100µl injection was made into the left knee joint. Weight distribution and PAM withdrawal thresholds were measured every 2 hours after surgery for 7 hours and then daily, along with body weights and knee joint diameters. 2-way ANOVAs were used to assess statistical significance, the null hypothesis was rejected at $P<0.05$.

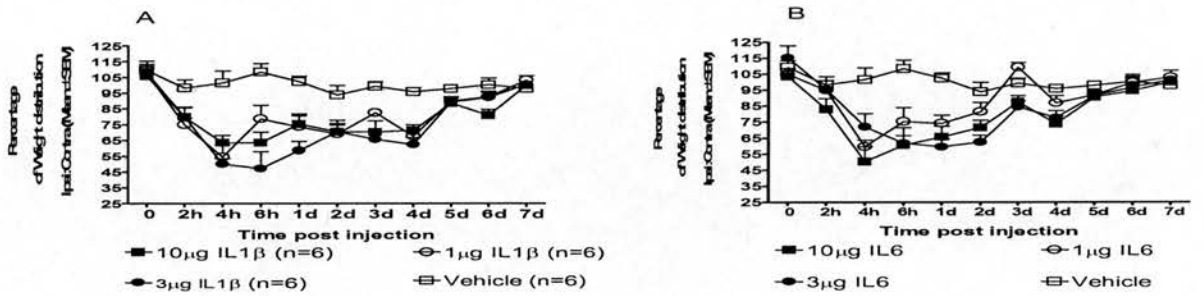


Figure 1. The weight distribution of the ipsilateral hind limb expressed as a percentage of the contralateral limb for (A) IL1β and vehicle and (B) IL6 and vehicle. When compared to vehicle, all IL1β doses were significantly lower at t=4h, 6h, 1d, 2d and 4d, whilst 10 and 3µg IL1β were also significant at t=3d. All doses of IL6 were significantly lower than vehicle at t=4h, 6h and 1d; 3µg was also significant at t=2d.

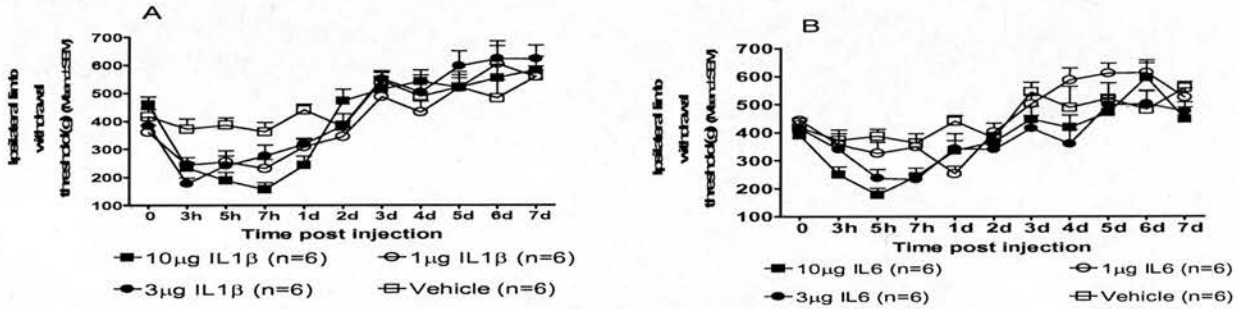


Figure 2. The ipsilateral limb withdrawal thresholds for (A) IL1β and vehicle and (B) IL6 and vehicle. When compared to vehicle, 10µg IL1β was significantly reduced at t=5h, 7h and 1d; 3µg Il1β was significant at t=3h; 10µg IL6 was significant at t=5h and 1µg IL6 was significant at t=1d.

There were no significant differences between the body weights, ipsilateral or contralateral joint diameters or contralateral PAD withdrawal thresholds of any group at any time point. This study shows that both IL1β and IL6 can cause hypersensitivity and allodynia acutely and that the decrease in ipsilateral withdrawal threshold resulting from either cytokine is short-lived and has returned to baseline after 1day. In contrast, the hypersensitivity of the joint injected with IL1β or IL6, as measured with weight distribution, is more sustained, but that IL1β-induced hypersensitivity has a longer duration than that induced by IL6. However, neither cytokine induces joint swelling.

Clayton, N.M. *et al.* (1997) *Br J Pharmacol,* **120**, 75P

Eastgate *et al.* (1988) Lancet, **2,** 706-9

Houssiau *et al* (1988) Arthritis Rheum, **31,** 784-8

Strickland *et al.* (2005) pA2 Online, In Press.

Intra-articular IL1β or IL6 induce acute mechanical hypersensitivity and allodynia in the rat knee joint

N.J.Barton[1], I.T.Strickland[1], I.P.Chessell[2], A.J.Reeve[2], D.S.McQueen[1]

[1]School of Biomedical Sciences, College of Medicine & Veterinary Medicine, University of Edinburgh, 1 George Square, Edinburgh, EH8 9JZ, UK
[2]Neurology & Gastrointestinal CEDD, GlaxoSmithKline, Harlow, CM19 5AW, UK

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease characterised by persistent synovitis that results in progressive joint destruction. It affects approximately 1% of the general population. Most drugs prescribed for RA are non-steroidal anti-inflammatory drugs (NSAIDs), to reduce inflammation and the associated pain. Recently disease-modifying anti-rheumatic drugs (DMARDs), including anti-cytokine drugs, have shown promising effects clinically.

Interleukin (IL) 1β and IL6 are pro-inflammatory cytokines present in high levels in rheumatoid arthritic joints; in addition their blood concentrations correlate with rheumatoid arthritis disease activity (Eastgate *et al.* 1988; Houssiau *et al.*1988).

2. Aim

The aim of this experiment was to determine whether intra-articular injections of IL1β or IL6 cause hypersensitivity or allodynia in the rat knee (stifle) joint.

3. Methods

•Experiments were performed in accordance with Home Office regulations and within UK animal welfare guidelines, and received Local Ethics Committee approval.

Body weights, knee joint diameters (a measure of swelling; measured using digital microcallipers), the weight distribution between hind limbs (a measure of hypersensitivity; Clayton *et al.*, 1997; Linton Instruments) and the withdrawal threshold to a gradually increasing localised pressure applied to the knee joint (a measure of allodynia; Strickland *et al.* 2005; PAD), were measured in 42 male Wistar rats (150-175g). Animals were divided into seven groups, which were injected with either 1, 3 or 10μg IL1β or 1, 3 or 10μg IL6 (rat recombinant; Bioclone Inc, USA) or vehicle (sterile saline). Rats were transiently anaesthetised with 3% halothane in O_2 and a 100μl injection was made into the left knee joint (i.art). Weight distribution and PAD withdrawal thresholds (WT) were measured every 2 hours after surgery for 7 hours and then daily, together with body weights and knee joint diameters. 2-way ANOVAs were used to assess statistical significance between group means, the null hypothesis was rejected at $P<0.05$.

4. Results – Joint Swelling

Neither IL1 β or IL6 had any effect on ipsilateral or contralateral joint diameters, a measure of swelling, at any time point during the study compared to vehicle-treated animals ($P>0.05$).

5. Results – Mechanical Hypersensitivity

Both i.art IL1β and IL6 evoked a mechanical hypersensitivity which was sustained for 3-4 days.

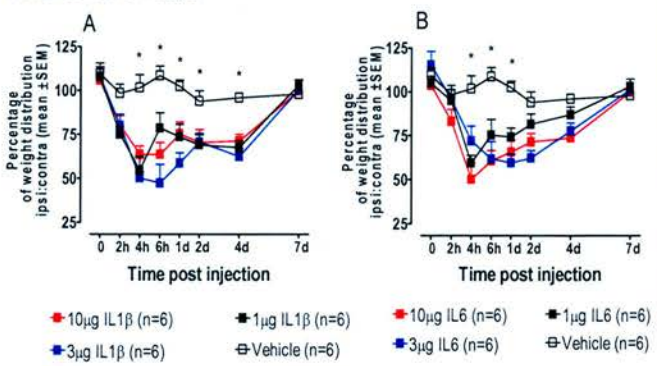


Figure 1. The weight distribution of the ipsilateral hind limb expressed as a percentage of the contralateral limb for (A) IL1β-injected- and vehicle-injected animals and (B) IL6-injected- and vehicle-injected animals. When compared to vehicle, the percentage weight distribution between the ipsilateral and contralateral limbs in all IL1β-injected groups was significantly reduced at 4h, 6h, 1day (d), 2d and 4d ($P<0.05$). The percentage of weight distribution in all groups injected with IL6 was significantly lower than vehicle at 4h, 6h and 1d ($P<0.05$); 3μg was also significantly lower at 2d ($P<0.05$).

6. Results - Allodynia

Both i.art IL1β and IL6 caused a reduction in the ipsilateral limb withdrawal thresholds to a gradually increasing localised pressure applied across the knee joint. However this effect was short-lived and only lasted up to 24 hours. The effect was more pronounced in animals injected with IL1β than those treated with IL6.

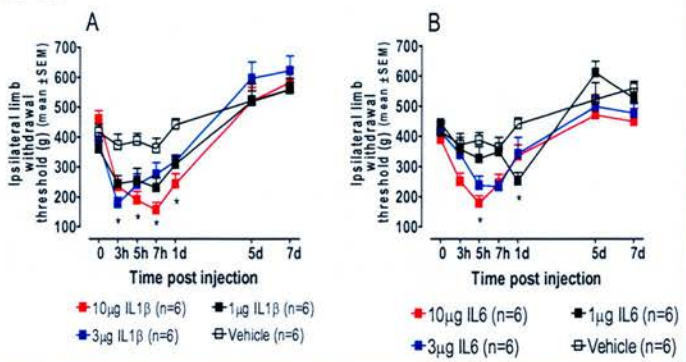


Figure 2. The ipsilateral limb withdrawal thresholds for (A) IL1β- and vehicle-injected animals and (B) IL6- and vehicle-injected animals. When compared to vehicle, the ipsilateral limb WT for the group injected with 10μg IL1β was significantly reduced at 5h, 7h and 1d ($P<0.05$); in addition, the ipsilateral WTs for animals injected with 3μg Il1β were significantly decreased at 3h ($P<0.05$). Animals injected with 10μg IL6 i.art had a significantly reduced ipsilateral WT at 5h ($P<0.05$) and those treated with 1μg IL6 had a significantly reduced WT compared to vehicle at 1d ($P<0.05$).

7. Summary and Conclusions

a. Neither IL1β or IL6 had any effect on joint swelling

b. This study shows that both IL1β and IL6 can induce mechanical hypersensitivity in the rat knee joint
   - IL1β caused a more sustained effect than IL6
   - The hypersensitivity is sustained for 3-4 days

c. Furthermore, both IL1β and IL6 can induce allodynia, as assessed by limb withdrawal threshold to a localised squeezing of the joints
   - This effect by i.art IL1β and IL6 was short-lived
   - The withdrawal threshold returned to baseline within 24hours

d. These results suggest that IL1β and IL6 may be key mediators in the development of pain associated with inflammatory joint diseases.

Clayton, N.M. *et al.* (1997) *Br J Pharmacol*, **120**, 75P
Eastgate *et al.* (1988) Lancet, **2**, 706-9
Houssiau *et al* (1988) Arthritis Rheum, **31**, 784-8
Strickland *et al.* (2005) pA2 Online, In Press

A novel behavioural readout for assessing hypersensitivity of knee joints in a murine model of unilateral arthritis

I.T.Strickland[1], N.J.Barton[1], H.M.Brash[1], D.S.McQueen[1], A.J.Reeve[2], A.W.Wilson[2], I.P.Chessell[2]

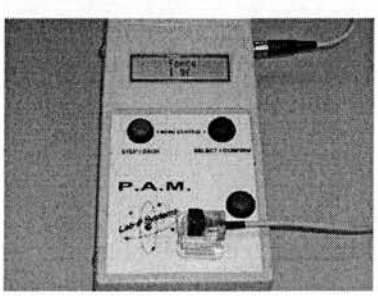
[1] Division of Neuroscience, College of Medicine and Veterinary Medicine, University of Edinburgh, 1 George Square, Edinburgh, EH8 9JZ.

[2] Neurology & Gastrointestinal CEDD, GlaxoSmithKline, Harlow, UK, CM19 5AW

The aim of this study was to develop a new quantitative method for measuring hypersensitivity of the knee (stifle) joint during a murine model of unilateral arthritis (Gauldie *et al,*. 2004). The pressure application measurement device (PAM) is designed to objectively assess a discrete localised hypersensitivity across the knee joint. The force applied across a joint under test is measured by a force transducer fitted to the experimenter's thumb (see Figure 1). A gradually increasing 'squeeze' force was applied across the joint and by means of calibrated instrumentation the force in grams applied was recorded. The test endpoint was when the animal withdrew its limb. Male C57BL6 mice (n=24, 23-27g) were transiently anaesthetised (3% isofluorane with O_2 at $1.5l.min^{-1}$) and a small incision was made over the knee joint to show the patella tendon, sham (n=8) animals recovered with no further treatment. The remaining 16 animals were injected with Freund's complete adjuvant (FCA; 20µl, $10mg.ml^{-1}$) under the patella tendon into the intra-articular space of the joint. Prior to surgery and for 21 days following PAM withdrawal threshold, weight bearing (Clayton *et al,*. 1997), knee diameter and body weight were recorded. On day 10 the FCA injected animals were ranked and randomised into two matched groups (n=8) and dosed (s.c.) on days 13-17 with either prednisolone ($1mg.kg^{-1}$) or vehicle (ethanol 5%, PEG 45% and distilled water 50%). PAM data were transformed into an area under the curve (AUC) value and analysed using a Mann-Whitney test, where $p<0.05$ was considered significant. Results showed FCA injected animals had significantly lower withdrawal thresholds than sham on day 3 ($p= 0.0001$), when compared to day 0, confirming the FCA induced hypersensitivity. Over days 13-17 the prednisolone treated group had significantly higher AUC values than the vehicle group ($p= 0.0104$), compared to day 10, due to a reversal of hypersensitivity. PAM results correlated significantly with weight bearing data ($r= 0.9$, Pearson's correlation). These results show that PAM is a robust tool which provides an easy to use objective method for localised assessment of knee joint hypersensitivity in mice, which correlates strongly with a previously accepted behavioural readout.

Clayton *et al,*. 1997 *Br J Pharmacol* **120**:219P.
Gauldie *et al,*. 2004 *J Neurosci Methods* **139**(2):281-291.

Figure 1. Pressure application measurement device (PAM)



A novel behavioural readout for assessing hypersensitivity of knee joints in a murine model of unilateral arthritis

I.T.Strickland[1], N.J.Barton[1], H.M.Brash[1], D.S.McQueen[1], A.J.Reeve[2], A.W.Wilson[2], I.P.Chessell[2]

[1] Division of Neuroscience, College of Medicine and Veterinary Medicine, University of Edinburgh, 1 George Square, Edinburgh, EH8 9JZ

[2] Neurology & Gastrointestinal CEDD, GlaxoSmithKline, Harlow, UK, CM19 5AW

Aim

The aim of this study was to develop and validate a new quantitative method for measuring hypersensitivity of the knee (stifle) joint in a murine model of unilateral arthritis (Gauldie *et al,*. 2004).

Introduction

Several behavioural readouts are currently used to assess the hypersensitivity in the joints of arthritic rodents (Clayton *et al,*. 1997). However none of these are able to assess a localised hypersensitivity directly across the arthritic joint. The pressure application measurement (PAM) device measures and records the force applied across a joint under test using a force transducer fitted to the experimenter's thumb.

Apparatus

PAM apparatus includes a base unit with display screen (Fig.1A) and a force transducer, with 5mm diameter application surface, mounted on a unit worn on the experimenters thumb. Placing the force transducer on one side of the joint and the forefinger on the opposite (Fig. 1B), a gradually increasing 'squeeze' was applied in a mediolateral plane across a joint. By means of the calibrated instrumentation the force in grams (gf) applied was recorded (max = 500gf). The test endpoint was when the animal withdrew its limb. The measured forces are quoted in gf (gram force) and may be converted to N (Newton) by multiplying by 9.8159×10^{-3}

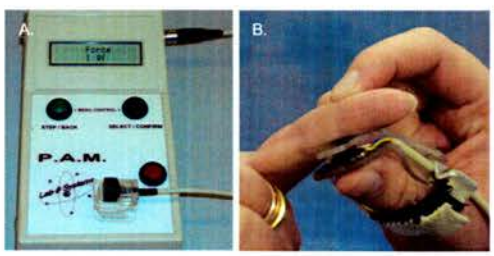


Fig. 1. **(A)** PAM device showing the base unit with force display screen and thumb worn force transducer. **(B)** Example of how the gradually increasing 'squeeze' force is applied across a joint. The transducer is placed on one side of the joint and the forefinger on the opposite.

Methods

Weight distribution over the hind limbs (Clayton *et al,*. 1997), PAM, knee joint diameters and body weights were measured in male C57BL6 mice (n=24, 23-27g) for a period of 21 days. Following day 0 recordings the mice were transiently anaesthetised (3% isofluorane with O_2 at 1.5l.min^{-1}) and a small incision was made over the knee joint to expose the patella tendon, sham (n=8) animals recovered with no further treatment. The remaining 16 animals were injected with Freund's complete adjuvant (FCA; 20µl, 10mg.ml^{-1}) under the patella tendon into the intra-articular space of the knee joint (Gauldie *et al,*. 2004).

On day 10 the FCA injected animals were ranked and randomised into two matched groups (n=8 per group), based on their level of withdrawal threshold (gf) as recorded by PAM. The groups were dosed (s.c.) on days 13-17 blindly with either prednisolone (1mg.kg^{-1}) or vehicle (ethanol 5%, PEG 45% and distilled water 50%) one hour before recordings were taken. All procedures were in accordance with U.K Home Office Regulations.

Data were transformed into an area under the curve (AUC) value during the dosing period and analysed using a Mann-Whitney test, where $P<0.05$ was considered significant.

Results

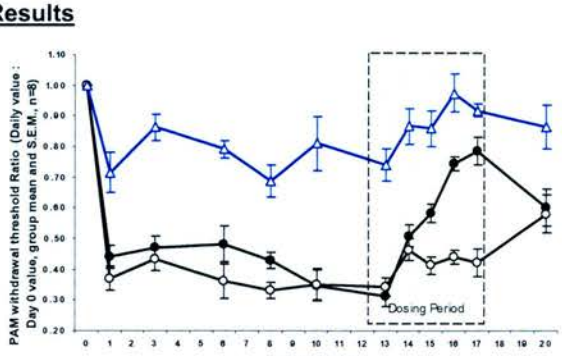


Fig. 2. Effect of prednisolone (n=8, s.c., 1mg.kg^{-1}, filled circle) or vehicle (open circle) on the FCA induced hypersensitivity (n=16, I.art., 20µl, all circles) over days 13-17 as measured by the PAM device. Untreated sham (n=8) animals are represented by open triangles. Day 0 ratios of 1.0 are based on the day 0 group mean withdrawal threshold values of 402gf, 400gf and 437gf.

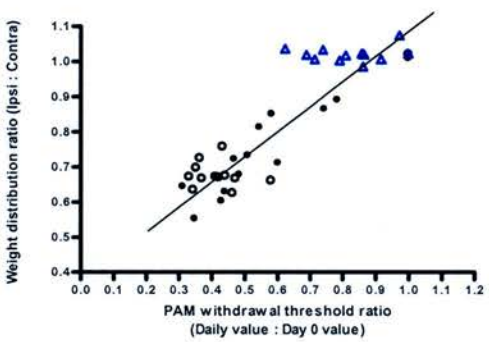


Fig. 3. Graph showing the correlation between the group mean weight distribution ratio (Ipsilateral : Contralateral) and the group mean PAM withdrawal threshold ratio (Daily value : Day 0 value) for days 0-20. Groups are represented as follows; sham (open blue triangles), FCA + vehicle (open black squares) and FCA + prednisolone (s.c., 1mg.kg^{-1}, closed black circle).

- Results showed FCA injected animals had significantly lower withdrawal thresholds than sham on day 3 (P= 0.0001), when compared to day 0, confirming the FCA induced a localised hypersensitivity which was detected using the PAM device.
- During the treatment period (days 13-17) the prednisolone treated group had significantly higher AUC values, compared to day 10, than the vehicle group (P= 0.0104). This was due to a reversal of hypersensitivity by prednisolone which was also observed using the PAM device.
- A strong correlation between the PAM device and weight distribution results was observed (Pearson's r correlation = 0.9).

Summary and Conclusions

- PAM is a robust instrument which provides an easy to use, objective method for localised assessment of knee joint hypersensitivity in mice and correlates strongly with a previously accepted behavioural readout.
- A second PAM device is currently under development designed and calibrated for measuring hypersensitivity in rat knee joints.

Acknowledgements

Dr. Harry Brash for his hard work in designing and building PAM. The work was funded by GlaxoSmithKline and the BBSRC as part of PhD studies (ITS and NJB).

References

Clayton *et al,*. 1997 *Br J Pharmacol* **120**:219P.
Gauldie *et al,*. 2004 *J Neurosci Methods* **139**(2):281-291.6

Available online at www.sciencedirect.com
ScienceDirect
Experimental and Molecular Pathology 81 (2006) 166–170
Experimental and Molecular Pathology
www.elsevier.com/locate/yexmp

Attenuation of experimental arthritis in TRPV1R knockout mice

N.J. Barton [a,*], D.S. McQueen [a], D. Thomson [a], S.D. Gauldie [a], A.W. Wilson [b], D.M. Salter [c], I.P. Chessell [b]

[a] *Division of Neuroscience, University of Edinburgh, Medical College, 1 George Sq, EH8 9JZ, UK*
[b] *Neurology CEDD, GlaxoSmithKline R&D Ltd, Harlow, Essex CM19 5AW, UK*
[c] *Pathology, Queen's Medical Research Institute, University of Edinburgh, Medical College, Little France, Edinburgh, EH16 4TJ, UK*

Received 24 April 2006
Available online 16 June 2006

Abstract

The Transient Receptor Potential Vanilloid 1 (TRPV1R) is a ligand-gated, non-selective cation channel expressed predominantly by sensory neurons. TRPV1Rs respond to a variety of noxious stimuli including capsaicin, intense heat and acid. These factors, combined with behavioral studies, show that TRPV1Rs are involved in nociception. The aim of our study was to determine whether TRPV1Rs play a role in the development and maintenance of inflammation and mechanical hyperalgesia by studying the development of unilateral joint inflammation in TRPV1R−/− mice. Knee joints of TRPV1R−/− or wild-type (WT) mice were injected with FCA (200 μg) under temporary anesthesia, and the resulting inflammation and hyperalgesia measured for 35 days. Histological analysis was performed on joints at the end of the study. TRPV1R−/− mice developed mild joint swelling which was significantly less than that obtained in WT mice ($P < 0.05$, Mann–Whitney). The ratio of the weight distribution between the hind limbs in TRPV1R−/− mice was also significantly less than in WT mice ($P < 0.05$, Mann–Whitney). Neither swelling nor hypersensitivity was completely absent in the knockout mice, indicating either that other mechanisms are involved or that a compensatory mechanism operates in TRPV1R−/− mice. These results suggest that TRPV1 receptors are important for the development of joint inflammation and the associated mechanical hypersensitivity observed in this model.

Keywords: Arthritis; FCA; TRPV1; Inflammation; Mechanical hyperalgesia

Introduction

The Transient Receptor Potential Vanilloid 1 receptor (TRPV1R; previously known as VR1 or capsaicin receptor) is a ligand-gated, non-selective cation channel expressed predominately by primary nociceptive sensory neurons (Caterina et al., 1997). It is found on nerves that innervate the skin (Foster and Ramage, 1981), cornea (Belmonte et al., 1991), mucous membranes of the mouth (Szolcsanyi and Jancso-Gabor, 1975), muscles (Kaufman et al., 1982), joints (He et al., 1988) and in several visceral organs within the cardiovascular, respiratory and genitourinary systems (Coleridge and Coleridge, 1977; Maggi et al., 1986). In addition, these receptors are located in the central nervous system, on small and medium size dorsal root ganglion (DRG) cells (Caterina et al., 1997; Guo et al., 1999; Helliwell et al., 1998), which co-express isolectin B4 (IB4), P2X3 purinoceptors or neuropeptides (Guo et al., 1999). Furthermore, TRPV1Rs are located on central processes of DRG cells, in particular, those in superficial laminae of the dorsal horn (Szallasi, 1995). The TRPV1R is a polymodal detector of noxious stimuli including capsaicin, the pungent ingredient in chili peppers, heat above 43°C and extracellular pH <6 (Caterina et al., 1997). It is also capable of integrating simultaneous exposure to these stimuli (Tominaga et al., 1998), making it of fundamental importance in the transduction of many types of nociceptive input (Szallasi and Blumberg, 1999).

It has been reported that mice lacking TRPV1Rs are less responsive to noxious heat and do not develop inflammation-

Abbreviations: ANOVA, analysis of variance; CGRP, calcitonin-gene-related peptide; DRG, dorsal root ganglion; EDTA, ethylenediaminetetraacetic acid; FCA, Freunds Complete Adjuvant; IB4, isolectin B4; PMN, polymorphonuclear neutrophils; SEM, standard error of the mean; TRPV1R, Transient Receptor Potential Vanilloid 1 receptor; WT, wild type.

* Corresponding author. Fax: +44 131 651 1835.
E-mail address: N.J.Barton@sms.ed.ac.uk (N.J. Barton).

0014-4800/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2006.04.007

induced thermal hyperalgesia (Caterina et al., 1997). A selective reduction in thermal nociceptive input to the spinal cord dorsal horn (evoked by heating of hind paw) has also been reported, although this was not the case following partial ligation of the sciatic nerve (Caterina, 2003). TRPV1R knockout mice show selective deficits in behavioral assays, including tail immersion, hot plate and radiant heating of the paw. Heat-evoked withdrawal at relatively low noxious temperatures was comparable to that of wild-type littermates, while at temperatures above 43°C TRPV1R−/− mice displayed a longer withdrawal latency compared to normal mice, consistent with impaired thermal nociception (Caterina et al., 2000). This evidence suggests that TRPV1Rs are involved in the peripheral sensitization of nociceptors that occurs following inflammation. In addition, small diameter DRG neurons isolated from TRPV1R−/− mice lacked many of the capsaicin-, acid- and heat-gated responses that have been well characterized in these neurons (Davis et al., 2000). In the intact mouse, these deficits manifest as reductions in paw licking and inflammation evoked by intraplantar injection of either capsaicin or resiniferatoxin (Caterina et al., 2000), demonstrating that TRPV1Rs are essential for vanilloid-evoked inflammation and nociception.

Caterina et al. (2000) reported that there was no decrease in mechanically evoked input to the spinal dorsal horn in TRPV1R−/− mice following hind paw inflammation. However, no studies to date have investigated the role of the TRPV1R on mechanical hyperalgesia and inflammation following experimentally induced murine arthritis, a joint inflammation. The aim of our study was to test the hypothesis that TRPV1R on nociceptive sensory nerves is crucial for the development and maintenance of mechanical hyperalgesia and joint inflammation in a unilateral model of chronic murine arthritis induced by FCA. TRPV1 antagonists may have utility as novel analgesics and hypothetically could deliver efficacy with a safety profile quite different from the cyclooxygenase-2 inhibitors, some of which carry a potential long-term cardiovascular liability.

Materials and methods

Experiments were performed in accordance with Home Office Regulations and within UK animal welfare guidelines and received Local Ethics Committee approval. Nine male C57 Black 6 (C57BL/6; Charles River, UK) wild-type (WT) and 20 C57BL/6 male TRPV1R−/− (GlaxoSmithKline, Harlow, UK) mice (weight range: 15–25 g) were used to investigate the consequence of TRPV1R−/− on the development of unilateral chronic joint inflammation. Animals were housed four to a cage in a 12-h light/dark environment and were given free access to standard animal feed and water for the duration of the study.

Knockout of TRPV1R gene

Homologous recombination in embryonic stem cells was used to generate a mouse lacking transmembrane domains 2–4 of the TRPV1R gene (i.e. DNA encoding amino acids 460–555) at GlaxoSmithKline (Harlow, UK; Davis et al., 2000). Ten male heterozygous (TRPV1R−/+) mice were mated with female TRPV1R+/+ WT C57BL/6 mice, giving rise to overtly healthy litters in the expected Mendelian ratios. Homozygous TRPV1R−/− mice were then generated and crossed to provide a full colony of KO mice for the study. Genotypes of the mice were confirmed by polymerase chain reaction and gel electrophoresis, showing absence of the TRPV1R gene.

Induction of arthritis

Animals were injected with Freunds Complete Adjuvant (FCA; MAFF; 10 mg ml^{-1} *Mycobacterium tuberculosis* in paraffin oil plus mannide monooleate; $n = 10$ TRPV1R−/− and $n = 5$ WT) or vehicle (heavy liquid paraffin oil; $n = 10$ TRPV1R−/− and $n = 4$ WT; Gauldie et al., 2004). Briefly, injections were carried out by transiently anesthetizing the animal (3% halothane in oxygen) and making a small incision over the left knee joint to allow visual identification of the patella tendon. Using a 26-gauge needle mounted on a 50-μl Hamilton syringe, FCA or vehicle (20 μl) was injected under the patella tendon and directly into the synovial space of the knee joint.

Assessment of arthritis

Animals were weighed, and their knee joint diameters (left and right) were measured just below the level of the patella using hand-held micro-callipers (Mitutoyo, Japan; accuracy ±0.1 mm).

Weight distribution between the animal's hind limbs was measured using an Incapacitance Tester (Linton Instruments; Clayton et al., 1997), which provides a measure of hyperalgesia. Measurements were made three times per week by the same operator.

Histopathology

Animals were killed by cervical dislocation at the end of the study (day 35), and the left and right knee joints were removed for histology. Joints were excised by cutting through the femur and tibia with the skin intact and were then fixed in 30 ml of 10% neutral buffered formalin (Sigma) for 3 days. Joints were decalcified in a saturated solution of EDTA (ethylenediaminetetraacetic acid) for up to 3 weeks, processed into paraffin wax and 4 μm sections were cut and stained with either hematoxylin and eosin or alcoholic toluidine blue. Slides were then viewed and photographed.

Data analysis and statistics

Results were collated and analyzed using Graph pad Prism and Microsoft Excel software. Unpaired non-parametric tests were used to analyze the difference between means of two independent groups. For paired data, the Mann–Whitney test (non-parametric) was used; for non-paired data, the Wilcoxon test (non-parametric) was used. To determine differences between the means of more than two groups, a two-way analysis of variance (ANOVA) was performed and a post hoc test was performed if the result was significant (Bonferroni multiple comparison). A P value of less than 0.05 was considered significant, rejecting the null hypothesis that the variation was due to chance. All data are expressed as mean ± standard error of the mean (SEM).

Results

TRPV1R-null mice were viable and fertile and exhibited a normal appearance and behaviors

Intra-articular injection of FCA had no adverse effects in either group of mice as animals continued to feed and gain weight normally throughout the study. There was no significant difference in body weight between FCA-injected and vehicle-injected WT or TRPV1R−/− mice on days 0, 7, 14, 21 and 28 post-FCA ($P > 0.05$, ANOVA; data not shown).

Development of unilateral arthritis in TRPV1R−/− mice and WT mice after FCA

Baseline ipsilateral knee joint diameters in WT and TRPV1R−/− mice averaged 3.6 ± 0.2 mm and 3.8 ± 0.2 mm

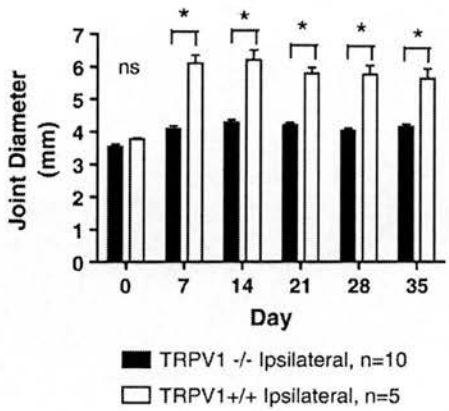


Fig. 1. Ipsilateral inflammation of the knee joint in TRPV1R−/− and WT mice following FCA. The ipsilateral inflammation, as assessed by swelling, was significantly less in TRPV1R−/− mice compared to WT on each day post-FCA ($P < 0.05$, ANOVA). There was no significant difference between the ipsilateral joints of the two groups of mice on day 0. Each bar represents the mean ± SEM. Statistical significance is represented by *. To determine differences between the means of the ipsilateral joint diameters in the 2 groups of mice, on each day, a two-way analysis of variance (ANOVA) was performed and a post hoc test was performed if the result was significant (Bonferroni multiple comparison).

respectively; these values were not significantly different ($P > 0.05$, Wilcoxon).

An intra-articular injection of FCA into the knee joint induced inflammation in C57BL/6 WT ($n = 5$) and TRPV1R−/− ($n = 10$) mice, but the response was significantly attenuated in TRPV1R−/− mice ($P < 0.05$, ANOVA); this was the case at all time points in the study (see Fig. 1).

Contralateral joint diameters were not significantly different from their day 0 values at any time point, either in the WT or the TRPV1R−/− mice, and there was no significant difference between the WT and TRPV1R−/− contralateral joint diameters ($P > 0.05$, ANOVA).

The joint diameter in the TRPV1R−/− mice was significantly increased compared to day 0, when measured on days 7, 14, 21 and 35 ($P < 0.05$, Mann–Whitney). However, the swelling in the ipsilateral knee joints of TRPV1R−/− mice was significantly less than that observed in WT. The ipsilateral joint's diameter increased by 15.5, 21.1, 18.3 and 16.9% on days 7, 14, 21 and 35 respectively. The ipsilateral FCA-injected TRPV1R−/− joint diameter on day 28 did not differ significantly from the corresponding pre-FCA value ($P > 0.05$, Mann–Whitney). In contrast, the ipsilateral joint of WT mice increased in diameter by 61.6, 64.0, 53.4, 52.3 and 48.1% on days 7, 14, 21, 28 and 35 respectively, all of which were significantly different from day 0 measurements ($P < 0.05$, Mann–Whitney).

Vehicle-injected joints showed no significant inflammation in either TRPV1R−/− ($n = 10$) or WT mice ($n = 4$) at any time point, as compared to the baseline values ($P > 0.05$, Mann–Whitney; data not shown). Contralateral joints of vehicle-injected mice showed no significant difference at any time point post-injection of FCA ($P > 0.05$, ANOVA; data not shown).

Incapacitance following induction of unilateral arthritis by FCA in TRPV1R−/− and WT mice

Prior to FCA injection (day 0), the ratio of weight distribution between ipsilateral and contralateral limbs was 1.0 ± 0.05 in TRPV1R−/− mice ($n = 10$) and 0.94 ± 0.09 in WT mice ($n = 5$), and these values were not significantly different ($P < 0.05$, Mann–Whitney).

The ratio of weight distribution between the hind limbs for TRPV1R−/− mice decreased by 35% (to 0.65 ± 0.04), 25% (to 0.75 ± 0.14), 17% (to 0.83 ± 0.08), 12% (to 0.88 ± 0.1) and 6% (0.94 ± 0.04) on days 7, 14, 21, 28 and 35 respectively, all of which were significantly different from the value on day 0 ($P < 0.05$, Mann–Whitney). In contrast, there was a significantly greater change in weight distribution on the hind limbs in WT mice than in TRPV1R−/− joints at all time points ($P < 0.05$, Wilcoxon test). From 0.94 ± 0.08, there was a decrease of 67, 60, 45, 44 and 45% on days 7, 14, 21, 28 and 35 respectively, all of which were significantly different from day 0 ($P < 0.05$ Mann–Whitney; see Fig. 2).

Intra-articular injection of vehicle into the left knee joint in TRPV1R−/− mice ($n = 10$) and WT mice ($n = 4$) did not affect the weight distribution ($P > 0.05$ Mann–Whitney; data not shown). The ratios of weight distribution in FCA-injected TRPV1R−/− to vehicle-injected limbs were significantly different on days 7, 14, 21 and 28 post-injection ($P < 0.05$, ANOVA; data not shown). In addition, comparison of the ratio of weight distribution in FCA-injected WT to vehicle-injected WT showed a significant difference at all time points after FCA ($P < 0.05$ ANOVA, data not shown).

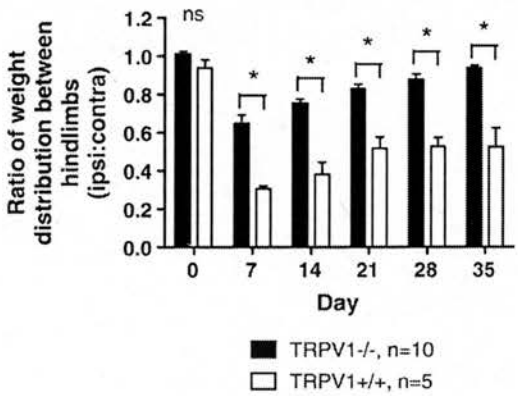


Fig. 2. The ratio of weight distribution on the ipsilateral and contralateral hind limbs for TRPV1R−/− mice and WT mice is shown. The difference between the ratios of the 2 groups of mice was statistically significant at all time points post-FCA ($P < 0.05$, ANOVA). TRPV1R−/− mice showed significantly less mechanical hypersensitivity than WT mice following joint injury, although they still exhibited some degree off hyperalgesia, as assessed by weight distribution. Each bar represents the mean ± SEM. Statistical significance is represented by *. To determine differences between the means of the ratios in the 2 groups of mice, on each day, a two-way analysis of variance (ANOVA) was performed and a post hoc test was performed if the result was significant (Bonferroni multiple comparison).

Histopathology

Pathological changes were observed 35 days after a single dose of FCA into the knee joint of WT and TRPV1R−/− mice. All FCA-treated animals had pathological changes in the injected (left) knee joint. In most cases, there was moderate hypertrophy of the synovial membrane and the infiltration of a large number of polymorphonuclear neutrophils (PMN) and macrophages into the joint space. There was also extensive inflammation in tissues outside the joint (peri-articular inflammation) including tendons, ligaments and surrounding muscle. In many, small amounts of pannus were observed associated with mild erosion of the articular cartilage and erosion of bone. Examples of new bone formation were also observed. In a few instances, mild pathology was noted, including mild thickening of the synovial membrane, PMN infiltration and peri-articular inflammation, in the absence of either joint swelling or hyperalgesia in vehicle-injected joints, likely a result of injury during injection. There were no differences detected between the TRPV1R−/− mice and WT mice. The contralateral knee joint showed no changes in any animal.

Discussion

This study was performed to determine whether TRPV1 receptors are essential for the development and maintenance of chronic unilateral arthritis in mice. Joint swelling, a measure of edema, was significantly greater in WT mice than it was in TRPV1R−/− mice following intra-articular FCA. Furthermore, mechanical hyperalgesia was reduced in knockout animals compared to WT.

The animal's weight when standing on the hind limbs was carried equally by both limbs in the normal state. Following injection of FCA, the weight distribution was altered. There was an increase in weight placed on the contralateral joint and a corresponding reduction in loading of the ipsilateral joint, meaning the inflamed limb was being guarded. TRPV1R−/− mice do not shift their weight to the contralateral limb to the same degree as WT mice, a finding that is consistent with reduced mechanical hyperalgesia.

Although the knee joints of knockout mice were inflamed following the induction of unilateral arthritis and were swollen in comparison with the contralateral limb or with vehicle-injected counterparts, the inflammation was significantly less than that developed by wild-type mice. This is in agreement with Keeble et al. (2004) who reported that TRPV1R knockout mice have decreased plasma extravasation in the knee joints following induction of experimental arthritis compared to wild-type mice. Caterina et al. (2000) also reported that TRPV1R−/− mice develop less paw swelling in response to intraplantar injection of vanilloid compounds.

The attenuated inflammation we observed in TRPV1R−/− mice in the present study probably resulted from a reduction in TRPV1R-induced peptide release from sensory afferent nerves. Thus, following stimulation by noxious mediators activated by injection of FCA, capsaicin-sensitive neurons trigger the release of neuropeptides from their peripheral terminals. Substance P (which triggers plasma extravasation) and calcitonin-gene-related peptide (CGRP; which stimulates vasodilatation) are the two main peptides released from capsaicin-sensitive sensory afferents; without the release of these peptides, the micro-environment following tissue injury may be quite different in TRPV1R −/− mice to that in wild-type mice, where release of peptides results in the recruitment of more serum factors and inflammatory cells to promote healing at the site of injury. However, under pathological circumstances, excessive neurogenic peptide release by capsaicin-sensitive afferent neurons is thought to contribute to the maladaptive inflammation associated with several conditions, including arthritis.

Davis et al. (2000) reported that knocking out the TRPV1 receptor eliminated thermal hyperalgesia following carageenan-induced inflammation. In addition, it has recently been demonstrated that TRPV1 receptors were responsible for post-operative heat hyperalgesia but were not involved in mechanical hypersensitivity in a model of incisional pain (Pogatzki-Zahn et al., 2005). However, this study also showed that neither the proportion of TRPV1R immunoreactive neurons in the DRG, nor the TRPV1R staining in the sciatic nerve, changed in WT mice following the tail incision. This is in contrast to other studies using other experimental models of pain. For example, Carlton and Coggeshall (2001) showed that TRPV1 receptors function and expression are increased in IB4-positive neurons following intraplantar FCA (Breese et al., 2005). Approximately 17% of unmyelinated C fibers in rat digital nerves immunostain for TRPV1R, and this proportion increased to almost 100% 48 h post-FCA inflammation, which would contribute to the thermal hyperalgesia and peripheral sensitization in inflammatory conditions (Carlton and Coggeshall, 2001). Other studies have shown that there were no differences between knockout and WT animals in formalin-induced nocifensive behavior, carageenan-evoked inflammatory mechanical hyperalgesia and partial sciatic nerve lesion-induced neuropathic mechanical hyperalgesia (Bolcskei et al., 2005).

Several other studies have shown that TRPV1 receptors are necessary for the development of thermal inflammatory hyperalgesia, but as yet, none has identified a role in joint mechanical hypersensitivity. We found that TRPV1R-null mice develop less mechanical hypersensitivity in the injected joint, as shown by the change in their weight distribution between hind limbs being less marked than in WT animals—suggesting less discomfort in the injected joint and a reduced mechanical hyperalgesia compared to wild-type mice. It has been reported that TRPV1R-null mice had normal withdrawal thresholds to punctuate stimuli applied to the hind paw. In addition, responses to intense tail pinch were also indistinguishable from those of wild-type littermates (Caterina et al., 2000). However, TRPV1R antagonists have been found to have analgesic activity in animal models of chronic inflammatory and neuropathic pain (Garcia-Martinez et al., 2002; Pomonis et al., 2003; Walker et al., 2003). Pre-treatment with the TRPV1 antagonist capsazepine prevented the development of mechanical hyperalgesia of capsaicin-injected foot pads in rats, mice and guinea pigs (Walker et al., 2003). In addition, capsazepine reversed mechanical hyperalgesia in the guinea pig FCA-injected hind

paw but had no effect in rats or mice (Walker et al., 2003). Similarly, in the partial sciatic nerve ligation model of neuropathic pain, capsazepine was surprisingly effective in the guinea pig, producing up to 80% reversal of mechanical hyperalgesia but had no effect in the rat or mouse (Walker et al., 2003).

It would be beneficial to have, in addition to the incapacitance readout, a measure of hyperalgesia present solely in the knee joint. This would enable the primary hyperalgesia in the joint itself, as a result of FCA-injection, to be studied; whereas the incapacitance tester measures whole limb changes. We are developing a device to measure the force applied to stifle joints, which will give significantly more information about hyperalgesia in the inflamed joint.

The histology showed that there were no major differences between the WT and TRPV1R−/− mice, which indicates that the TRPV1 receptor is not involved in initiating the pathological changes that occur within the joint. The receptor appears only to alter the hypersensitivity and the degree of inflammation that occurs alongside these structural changes.

In conclusion, our studies suggest that the TRPV1R plays an important role in the development and maintenance of inflammation of the joint and also in the mechanical hypersensitivity developed as a result of FCA injection. However, as the inflammation and hyperalgesia were not completely attenuated, it is probable that other mechanisms play a role in the inflammation processes and/or that, in the absence of TPRVR, other mediators and receptors may play a compensatory role. These results have implications for the future development of novel anti-arthritis drugs targeting the TRPV1 receptor as such agents may be analgesic as well as anti-inflammatory.

Acknowledgment

We thank GlaxoSmithKline for heterozygous knockout mice and for financial support for the study.

References

Belmonte, C., Gallar, J., Pozo, M.A., Rebollo, I., 1991. Excitation by irritant chemical substances of sensory afferent units in the cat's cornea. J. Physiol. 437, 709–725.

Bolcskei, K., Helyes, Z., Szabo, A., Sandor, K., Elekes, K., Nemeth, J., Almasi, R., Pinter, E., Petho, G., Szolcsanyi, J., 2005. Investigation of the role of TRPV1 receptors in acute and chronic nociceptive processes using gene-deficient mice. Pain 117, 368–376.

Breese, N.M., George, A.C., Pauers, L.E., Stucky, C.L., 2005. Peripheral inflammation selectively increases TRPV1 function in IB4-positive sensory neurons from adult mouse. Pain 115, 37–49.

Carlton, S.M., Coggeshall, R.E., 2001. Peripheral capsaicin receptors increase in the inflamed rat hindpaw: a possible mechanism for peripheral sensitization. Neurosci. Lett. 310, 53–56.

Caterina, M.J., 2003. Vanilloid receptors take a TRP beyond the sensory afferent. Pain 105, 5–9.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., Julius, D., 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389, 816–824.

Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., Koltzenburg, M., Basbaum, A.I., Julius, D., 2000. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288, 306–313.

Clayton, N.M., Oakley, I., Thompson, S., Wheeldon, A., Sargent, B., Bountra, C., 1997. Validation of the dual channel weight averager as an instrument for the measurement of clinically relevant pain. Br. J. Pharmacol. 120.

Coleridge, J.C., Coleridge, H.M., 1977. Afferent C-fibers and cardiorespiratory chemoreflexes. Am. Rev. Respir. Dis. 115, 251–260.

Davis, J.B., Gray, J., Gunthorpe, M.J., Hatcher, J.P., Davey, P.T., Overend, P., Harries, M.H., Latcham, J., Clapham, C., Atkinson, K., Hughes, S.A., Rance, K., Grau, E., Harper, A.J., Pugh, P.L., Rogers, D.C., Bingham, S., Randall, A., Sheardown, S.A., 2000. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 405, 183–187.

Foster, R.W., Ramage, A.G., 1981. The action of some chemical irritants on somatosensory receptors of the cat. Neuropharmacology 20, 191–198.

Garcia-Martinez, C., Humet, M., Planells-Cases, R., Gomis, A., Caprini, M., Viana, F., De La Pena, E., Sanchez-Baeza, F., Carbonell, T., De Felipe, C., Perez-Paya, E., Belmonte, C., Messeguer, A., Ferrer-Montiel, A., 2002. Attenuation of thermal nociception and hyperalgesia by VR1 blockers. Proc. Natl. Acad. Sci. U. S. A. 99, 2374–2379.

Gauldie, S.D., McQueen, D.S., Clarke, C.J., Chessell, I.P., 2004. A robust model of adjuvant-induced chronic unilateral arthritis in two mouse strains. J. Neurosci. Methods 139, 281–291.

Guo, A., Vulchanova, L., Wang, J., Li, X., Elde, R., 1999. Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. Eur. J. Neurosci. 11, 946–958.

He, X., Schmidt, R.F., Schmittner, H., 1988. Effects of capsaicin on articular afferents of the cat's knee joint. Agents Actions 25, 222–224.

Helliwell, R.J., McLatchie, L.M., Clarke, M., Winter, J., Bevan, S., McIntyre, P., 1998. Capsaicin sensitivity is associated with the expression of the vanilloid (capsaicin) receptor (VR1) mRNA in adult rat sensory ganglia. Neurosci. Lett. 250, 177–180.

Kaufman, M.P., Iwamoto, G.A., Longhurst, J.C., Mitchell, J.H., 1982. Effects of capsaicin and bradykinin on afferent fibers with ending in skeletal muscle. Circ. Res. 50, 133–139.

Keeble, J.E., Curtis, B., Mallaghan, F.A., Brain, S.D., 2004. The role of sensory nerves in joint inflammation: studies using TRPV1 knockout mice. pA2 online 2, 43P.

Maggi, C.A., Santicioli, P., Giuliani, S., Furio, M., Meli, A., 1986. The capsaicin-sensitive innervation of the rat urinary bladder: further studies on mechanisms regulating micturition threshold. J. Urol. 136, 696–700.

Pogatzki-Zahn, E.M., Shimizu, I., Caterina, M., Raja, S.N., 2005. Heat hyperalgesia after incision requires TRPV1 and is distinct from pure inflammatory pain. Pain 115, 296–307.

Pomonis, J.D., Harrison, J.E., Mark, L., Bristol, D.R., Valenzano, K.J., Walker, K., 2003. *N*-(4-Tertiarybutylphenyl)-4-(3-cholorphyridin-2-yl)tetrahydropyrazine-1(2*H*)-carbox-amide (BCTC), a novel, orally effective vanilloid receptor 1 antagonist with analgesic properties: II. in vivo characterization in rat models of inflammatory and neuropathic pain. J. Pharmacol. Exp. Ther. 306, 387–393.

Szallasi, A., 1995. Autoradiographic visualization and pharmacological characterization of vanilloid (capsaicin) receptors in several species, including man. Acta Physiol. Scand., Suppl. 629, 1–68.

Szallasi, A., Blumberg, P.M., 1999. Vanilloid (capsaicin) receptors and mechanisms. Pharmacol. Rev. 51, 159–212.

Szolcsanyi, J., Jancso-Gabor, A., 1975. Sensory effects of capsaicin congeners I. Relationship between chemical structure and pain-producing potency of pungent agents. Arzneim. -Forsch. 25, 1877–1881.

Tominaga, M., Caterina, M.J., Malmberg, A.B., Rosen, T.A., Gilbert, H., Skinner, K., Raumann, B.E., Basbaum, A.I., Julius, D., 1998. The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 21, 531–543.

Walker, K.M., Urban, L., Medhurst, S.J., Patel, S., Panesar, M., Fox, A.J., McIntyre, P., 2003. The VR1 antagonist capsazepine reverses mechanical hyperalgesia in models of inflammatory and neuropathic pain. J. Pharmacol. Exp. Ther. 304, 56–62.

ELSEVIER

Journal of Neuroscience Methods 163 (2007) 67–75

JOURNAL OF NEUROSCIENCE METHODS

www.elsevier.com/locate/jneumeth

Pressure application measurement (PAM): A novel behavioural technique for measuring hypersensitivity in a rat model of joint pain

Nicola J. Barton[a,1], Iain T. Strickland[a,*,1], Susan M. Bond[a], Harry M. Brash[b], Simon T. Bate[c], Alex W. Wilson[d], Iain P. Chessell[d], Alison J. Reeve[d], Daniel S. McQueen[a]

[a] *Division of Neuroscience, University of Edinburgh, Medical College, 1 George Square, Edinburgh EH8 9JZ, UK*
[b] *Department of Hepatology, University of Edinburgh, Royal Infirmary of Edinburgh, Little France, Edinburgh EH16 4SA, UK*
[c] *Statistical Sciences Europe, GlaxoSmithKline R&D Ltd., Harlow, Essex CM19 5AW, UK*
[d] *Neurology CEDD, GlaxoSmithKline R&D Ltd., Harlow, Essex CM19 5AW, UK*

Received 13 December 2006; received in revised form 2 February 2007; accepted 14 February 2007

Abstract

Chronic joint pain affects physical well being and can lead to severe psychological and social problems, therefore successful long-term management is highly sought-after. No current behavioural measures of pain used in pre-clinical models mimic the clinical dolorimeter, which provides an objective measure of joint hypersensitivity. In this study we aim to use a novel behavioural readout alongside an established measure to mimic the multifactorial measurements taken in the clinic. Using the pressure application measurement (PAM) device a gradually increasing squeeze was applied across the knee joint of rats until the animal gave an indication of pain or discomfort. PAM and the incapacitance tester were used to detect joint hypersensitivity in a well-established rodent model of adjuvant-induced arthritis. Subsequently, the analgesic effects of prednisolone (1, 3 or 10 mg kg^{-1}), morphine (3 mg kg^{-1}) and celecoxib (15 mg kg^{-1}) were assessed. Both PAM and the incapacitance tester detected a reversal of hypersensitivity 1 h post-drug administration. Furthermore, the two readouts were highly correlated, and power analysis indicated that PAM was highly reproducible. In conclusion, PAM provides a novel, accurate behavioural tool for detecting a primary mechanical hypersensitivity in a rat model of chronic inflammatory joint pain.

Keywords: Rat; Hypersensitivity; Joint; Force; Withdrawal; Pressure application

1. Introduction

Osteoarthritis (OA) and rheumatoid arthritis (RA) are prevalent diseases, with estimated incidences of 12.5% (Bedson et al., 2005) and 0.8% (Symmons, 2005) in the UK adult population, respectively. Arthritis is associated with chronic, debilitating pain in the joints. Pain is the most common symptom of patients seeking medical consultation (Loeser and Melzack, 1999). Chronic joint pain not only affects physical well being, for instance impairing the ability to work, but can also lead to severe psychological and social problems as a result of sleep disturbance, anxiety and depression (Ashburn and Staats, 1999). Successful long-term management is therefore highly sought-after.

Rodent models of chronic inflammatory joint hypersensitivity have been developed (Chillingworth and Donaldson, 2003; Donaldson et al., 1993; Gauldie et al., 2004; Wilson et al., 2006) to help elucidate the underlying pathophysiology involved in arthritic conditions by identifying specific key modulators or receptors involved in the pain process. These models are commonly used as pre-clinical screens for novel analgesics, with the aim to improve the treatment of chronic joint pain in the clinic. In order to do this successfully it is essential that results from pre-clinical models correlate well with observed changes in the clinic to facilitate the translation of novel treatments into successful patient pain management.

To date, several behavioural readouts have been used to assess joint hypersensitivity in animal models, using thermal, mechanical, or electrical sensory stimuli (for review see Le Bars et al., 2001). Only the weight distribution readout obtained using an incapacitance tester (Clayton et al., 1997) has so far been considered as a fair measurement of the persistent chronic hyper-

* Corresponding author. Tel.: +44 131 6511913; fax: +44 131 6511835.
E-mail address: s0093836@sms.ed.ac.uk (I.T. Strickland).
[1] Equal contributors.

0165-0270/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2007.02.012

sensitivity observed in patients. The incapacitance tester is able to provide an objective, non-evoked assessment of "incident" pain by measuring the average weight placed on each hind limb. Naïve rodents distribute their weight equally between both hind limbs under normal conditions (Kobayashi et al., 2003). However, following induction of joint inflammation, animals redistribute their weight in order to put less weight through the affected joint.

Whilst this readout has gone a long way to correlate with the clinical observation of 'pain on standing' it is only one measurement of complex pain. In the clinic, patients are scored by a variety of readouts or tasks, often being subjected to firm squeezing of affected joints (Ritchie et al., 1968). For instance, using the pressure dolorimeter (Langley et al., 1983), a localised quantitative measure of hypersensitivity directly around the affected joint can be measured. Using this device, a gradually increasing pressure is applied perpendicularly across the joint until the patient indicates tenderness or pain, at which point a 'sensitivity' score is recorded. Clinicians are able to summate a number of quantitative evaluations to assess a patient's level of pain or discomfort. In this study we aim to use a novel behavioural readout alongside an already established readout, with a view to bringing together two quantitative animal evaluations to mimic the multifactorial measurements taken in the clinic.

Here we describe a novel behavioural readout, for use with rats, utilising similar principles to the clinical pressure dolorimeter. The pressure application measurement (PAM) device is designed to apply a gradually increasing squeeze pressure directly across the knee joint of rats until the animal gives an indication of pain or discomfort. At this point a quantitative value of the force applied is recorded, giving a similar 'sensitivity' score.

The aim of this study was to determine if PAM was able to detect a Freund's complete adjuvant (FCA)-induced hypersensitivity in the knee joints of rats, and subsequently if PAM could detect a reversal of this hypersensitivity using analgesics known to be effective in the clinic. A dose–response study using prednisolone (Pyne et al., 2004), and further studies using morphine (Caldwell et al., 2002), and celecoxib (Schnitzer et al., 2005), were performed to investigate the reproducibility and sensitivity of PAM, whilst comparing the device with the already established weight distribution readout. Thus enabling an additional, clinically relevant pain readout to be applied pre-clinically aiding the translation of novel analgesics from animal to man.

2. Materials and methods

Experiments were performed in accordance with Home Office regulations and within UK animal welfare guidelines, and received Local Ethics Committee approval. Adult male Wistar rats (Charles River, UK; initial weight range 137–284 g) were used in the study. Animals were housed four to a cage in a 12 h light/dark environment and were given free access to standard animal feed and water for the duration of the study. All animals were allowed 6 days acclimatisation before the experiment began and all behavioural tests took place in the same room at the same time of day. Animals were weighed on each test day to monitor their general health.

2.1. Behavioural assessment

2.1.1. PAM–knee joint withdrawal threshold measurement

PAM consists of a force transducer mounted on a unit fitted to the operator's thumb (see Fig. 1). The thumb unit is connected to a recording base unit containing the control panel and digital readout display. The apparatus has a force transducer with a range of 0–1500 g (Honeywell, Farnell, UK) and the diameter of the circular contact is 8 mm; giving the device a surface area of 50.3 mm^2.

Animals were lightly, but securely held and the operator placed the thumb unit on one side of the animal's knee joint and the forefinger on the other. A gradually increasing squeeze force was applied across the joint at a rate of approximately 300 grams per second ensuring the maximum test duration was 5 s. By means of calibrated instrumentation the force in grams applied was displayed on the digital screen and recorded. The test endpoint was when the animal withdrew its limb or showed any behavioural signs of discomfort or distress, such as freezing of whisker movement or wriggling. As the animals were lightly

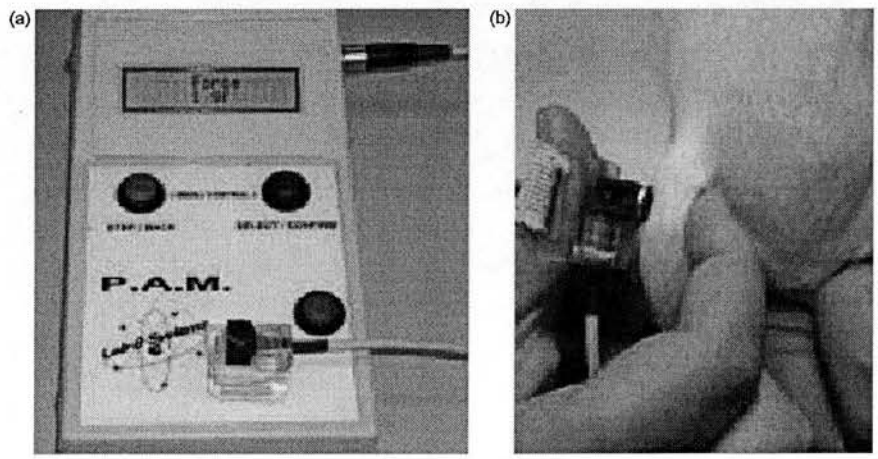


Fig. 1. Presentation of the pressure application measurement (PAM) device. (a) The portable control unit with digital display and thumb attachment; (b) PAM in use measuring the limb withdrawal threshold (LWT) of a rat.

held with their hind limbs suspended, any movements to pull or twist out of the operator's fingers were obvious. Any motion to attempt to withdraw from the device was transferred from the rat limb to the operator's fingers and forearm, and was taken as an indication of the test end point. On very rare occasions the first sign of distress was shown as the animal vocalising prior to limb withdrawal, and on these occasions this was taken as the test end point. The peak gram force (gf) applied immediately prior to limb withdrawal was recorded by the base unit, and this value was designated the limb withdrawal threshold (LWT). Three measurements of both the ipsilateral and contralateral limbs were made at 1 min intervals during which the animals were returned to their respective cages. The mean LWTs were calculated.

2.1.2. Incapacitance tester—weight distribution

The weight distribution between the animal's hind limbs was measured using the incapacitance tester (Linton Instruments, UK). The apparatus consists of two force transducers capable of measuring the body weight that the animal places on each hind limb. Animals were placed on the incapacitance tester with their hind paws centred on the two force transducers; the average body weight distribution in grams was calculated over a period of three seconds. Weight placed through the ipsilateral limb was expressed as a ratio of the weight placed through the contralateral limb, with a ratio of 1.0 resulting from equal weight distribution across both hind limbs. Each animal had three recordings taken per test day and the mean weight distribution ratio was calculated for each group.

2.1.3. Knee joint diameter

The knee joint diameters (left and right) were measured just below the level of the patella using hand-held digital micro-callipers (Mitutoyo, Japan; accuracy ±0.1 mm), used as a measure of swelling during the study.

2.2. Arthritis induction

Rats were transiently anaesthetised with 3% halothane in oxygen. FCA (1 mg ml^{-1}; Sigma, UK) was injected at a volume of 150 μl into the joint space of the left knee through the patella tendon using a sterile 25-gauge needle (B&D Microlance, UK). Control animals were anaesthetised but received no injection. The animals were allowed to recover from anaesthesia before being returned to cages.

2.3. Study design and drug treatment

A pilot study was carried out to determine whether PAM could detect a window of hypersensitivity following intra-articular FCA in comparison with sham animals. The study also compared values for PAM with those obtained in the same animals using the incapacitance tester. Baseline values for body weight, weight distribution, PAM LWT and knee joint diameters were measured on day 0, prior to induction of the joint inflammation, and were repeated two to three times per week until day 28. No habituation to the PAM device was conducted.

Secondly, a drug-treatment study was carried out, to investigate whether PAM was able to detect reversal of this joint hypersensitivity and how this differed with three types of drug, an opioid, a steroid and a non-steroidal anti-inflammatory drug (NSAID). In order to perform a thorough pharmacological investigation in this study, whilst using the minimum number of animals, a dose–response relationship to prednisolone was examined and single top doses of an opioid and an NSAID were also investigated. A pre-study behavioural assessment was introduced to habituate the animals to the device. Baseline values for body weight, weight distribution, PAM LWT and knee joint diameters were measured on day 0, prior to induction of the joint inflammation. Measurements were made two to three times per week until day 10, when animals were randomly assigned into treatment groups. Drugs were administered subcutaneously (s.c.) in a volume of 2 ml kg^{-1} between days 14 and 18. Prednisolone (1, 3 or 10 mg kg^{-1}) was dosed once per day; whereas morphine (3 mg kg^{-1}), celecoxib (15 mg kg^{-1}) and vehicle (ethanol 5%, PEG 45% and distilled water 50%) were given twice a day. The dose range of prednisolone used was decided from published results from the lab (Gauldie et al., 2004), and single doses of morphine and celecoxib were decided from other studies giving strong positive reversals of hypersensitivity (Wilson et al., 2006). One hour after blind dosing, behavioural assessments were made following a strict protocol in order to give animals a rest period between the weight distribution and the PAM measurements. Briefly, animals were weighed and had their weight distribution assessed by making three consecutive readings before being returned to their cage. Ten minutes later animals had the sensitivity of both the ipsilateral and contralateral joints assessed using PAM. Three consecutive readings were taken, with a 1 min interval between each measurement, where the animal was returned to the cage. Finally the knee joint diameters were measured. Further behavioural assessments were carried out once the dosing period was complete, on days 21, 24 and 28.

2.4. Data analysis

Prior to analysis, an area under the curve (AUC) summary of each animal's repeatedly measured responses during the dosing period was calculated. These AUC values were then analysed using a one-way analysis of variance (ANOVA) approach. Measurements taken over several days were analysed using a repeated measures ANOVA. In both cases, individual groups were compared using planned comparisons on the predicted means. Differences between ipsilateral and contralateral sides were analysed using paired *t*-tests. In all cases the null hypothesis was rejected at $P < 0.05$. The data were log transformed where appropriate to stabilize the variance. Data are expressed as observed mean ± standard error of the mean (SEM). Statistical analyses were carried out using Statistica version 6 (Statsoft, Tulsa, OK, USA).

Two different power analyses were carried out on the drug study to investigate the reliability and confidence levels obtained from the two different readouts in the framework of this experimental design. In the first, perhaps more standard power

analysis, the number of animals required to reach a certain power obtained from an observed drug effect was assessed. For this analysis, calculations were based on an effect size equivalent to the observed difference between vehicle and the highest dose of prednisolone.

In the second analysis, the effect of varying the number of animals used, or the numbers of measurements made, on the power of the experiment was assessed. By calculating the animal and measurement variance components, for the weight distribution and PAM LWT responses separately, we investigated the effect of varying replication of both animals and the measurements on the statistical power of the treatment comparisons.

3. Results

In both studies, intra-articular injection of FCA had no adverse effects on the animal's general health as evidenced by the fact that they continued to feed and gain weight normally throughout the study. There were no significant differences in body weights between any of the FCA-injected and sham animals at any time point (data not shown).

In both studies there were no significant differences between the mean PAM LWTs or knee joint diameters of the ipsilateral and contralateral limbs and no significant differences between treatment groups, prior to FCA injection on day 0 ($P > 0.05$).

3.1. Behavioural evaluation of FCA-induced hypersensitivity

3.1.1. Pressure application measurement (PAM)

Prior to induction of joint inflammation (on day 0), the average ipsilateral LWT was 710 ± 41 gf ($n = 16$) and the average contralateral LWT reading was 790 ± 39 gf ($n = 16$). Following injection of FCA, the average ipsilateral LWT of the treatment group decreased by 57% to 316 ± 45 gf ($n = 8$), by day 1. This was significantly lower than both the day 0 value ($P < 0.05$) and the sham group at the same time point ($P < 0.001$; see Fig. 2a). Despite receiving no further treatment the ipsilateral LWT of sham animals increased significantly by 57% to 1028 ± 48 gf ($n = 8$, $P < 0.05$) on day 1. The average ipsilateral LWT of FCA-injected rats were significantly lower than sham and contralateral joints ($P < 0.05$) over the full 28-day time course studied.

3.1.2. Incapacitance tester—weight distribution

The average ratio of weight distribution between ipsilateral and contralateral limbs in rats was 0.96 ± 0.03 ($n = 16$) on day 0. The ratio of weight distribution between the hind limbs for FCA injected rats decreased by 70% to 0.29 ± 0.05 ($n = 8$, see Fig. 2b). This was significantly less than sham animals ($P < 0.05$), which had a ratio of 1.05 ± 0.02 ($n = 8$) at this time point. The ratio remained significantly reduced in FCA-injected animals compared with the sham group up to and including day 28 ($P < 0.001$). There were no significant changes observed in the sham group at any time point in this behavioural readout.

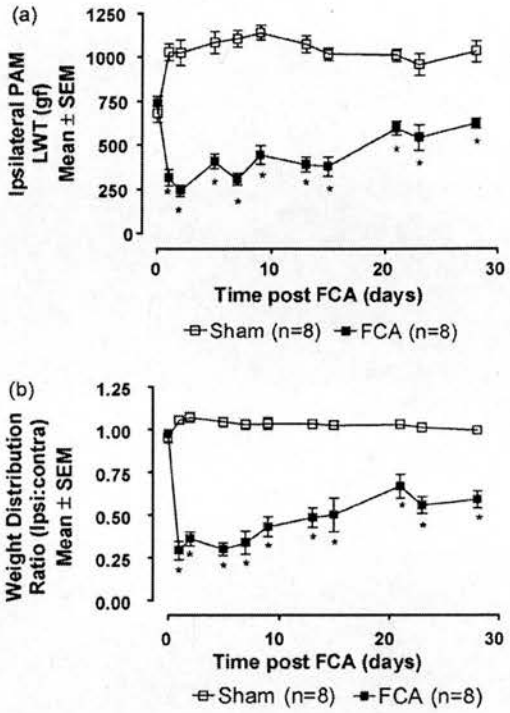


Fig. 2. (a) The ipsilateral PAM LWTs in gram force and (b) ratio of weight distribution between the hind limbs of sham ($n = 8$) and FCA-injected ($n = 8$) rats over a 28-day pilot study. PAM detected a large window of hypersensitivity, similar and comparable to that observed with the incapacitance tester. Statistical significance ($P < 0.05$) is represented by *; statistical analysis carried out to compare the two groups at each time point using a repeated measures ANOVA.

3.1.3. Joint inflammation

Basal measurements of joint diameter were 9.92 ± 0.08 mm for ipsilateral knees ($n = 16$), and 9.85 ± 0.12 mm for contralateral knees ($n = 16$). On day 1, the average ipsilateral knee joint diameter of FCA-injected animals was 13.11 ± 0.06 mm ($n = 8$), compared with 10.12 ± 0.1 mm ($n = 8$) in control animals. The swelling remained significant compared with sham animals on days 2, 5, 7 and 9 post-FCA ($P < 0.001$), however, thereafter the values returned towards basal levels, and no further significant differences were observed (data not shown).

3.2. Behavioural evaluation of the effect of a dose range of prednisolone, and single doses of morphine and celecoxib on the FCA-induced hypersensitivity

3.2.1. Pressure application measurement (PAM)

Measurements were made on days 0, 2, 4, 7 and 10, to determine that a window of hypersensitivity was present prior to drug treatment regimes. On each of these days the ipsilateral PAM LWTs of all FCA-injected animals ($n = 47$) were significantly less than those of the sham group ($n = 8$, $P < 0.0001$). On day 10, arthritic rats were randomly assigned into one of six treatment groups; celecoxib 15 mg kg^{-1} ($n = 8$), morphine 3 mg kg^{-1} ($n = 8$) or prednisolone 1 ($n = 7$), 3 ($n = 8$), or 10 mg kg^{-1} ($n = 8$). AUC values were calcu-

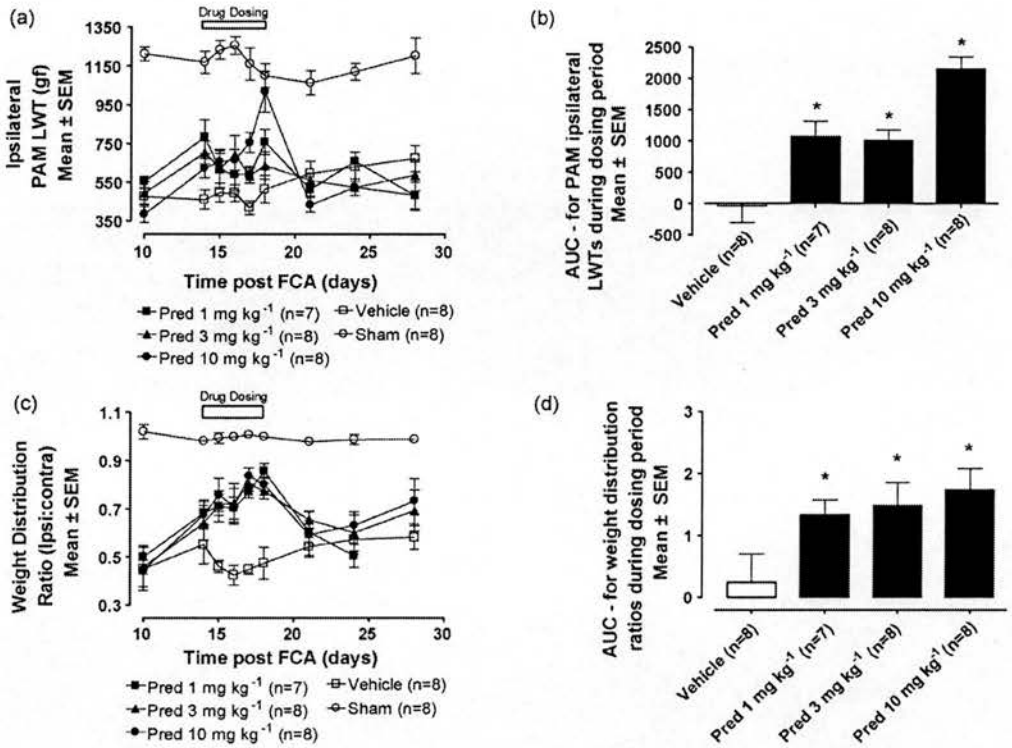


Fig. 3. The (a) ipsilateral PAM LWTs and (c) weight distribution ratios of vehicle and prednisolone treated arthritic rats and the corresponding graphs of AUC values for the two readouts during the dosing period (b and d). The drug-dosing period was between days 14 and 18 as indicated by the box on the graphs. The incapacitance tester was able to detect the dose-dependant reduction in hypersensitivity evoked by FCA in groups treated with 1, 3 and 10 mg kg^{-1} prednisolone. PAM did not show a three-point dose–response, instead the 1 and 3 mg kg^{-1} doses gave similar responses, whereas the 10 mg kg^{-1} gave a greater reversal. Statistical analysis was carried out to compare the AUC values for the drug-treated group compared with vehicle; *Statistical significance ($P<0.05$), determined by an ANOVA.

lated for each individual animal to observe any drug induced analgesia over the 5-day dosing period. AUC values were calculated using each animal's day 10 value as a baseline to observe changes from day 14 through to day 18. Results showed that PAM detected a significant analgesic effect of all drugs administered ($P<0.0001$ for morphine, celecoxib and prednisolone 10 mg kg^{-1}, and $P<0.05$ for prednisolone 1 and 3 mg kg^{-1}, compared with vehicle, see Figs. 3a and 4a).

Further PAM LWT measurements were taken after the cessation of drug-treatment, on days 21, 24 and 28. In all groups any analgesic effect of the compound previously administered was abolished, with there being no significant difference to the vehicle-treated group.

3.2.2. Incapacitance tester—weight distribution

Measurements were made on days 0, 2, 4, 7 and 10, to determine a window of hypersensitivity was present prior to drug treatment regimes. On each of these days the weight distribution ratio of all FCA-injected animals ($n=47$) was significantly less than those of sham ($n=8$, $P<0.0001$). AUC values were calculated for the dosing period and showed that the FCA-induced hypersensitivity was significantly reversed by celecoxib 15 mg kg^{-1} ($n=8$), morphine 3 mg kg^{-1} ($n=8$) and prednisolone at 3 ($n=8$) and 10 mg kg^{-1} ($n=8$) ($P<0.05$, compared with vehicle), but not by 1 mg kg^{-1} ($n=7$) prednisolone (see Figs. 3 and 4c). The weight distribution ratio AUC values also revealed a dose related reversal of hypersensitivity using the incapacitance tester (see Fig. 3d).

Further measurements made on days 21, 24 and 28, revealed that the incapacitance tester detected no lasting reversal of hypersensitivity following the cessation of drug administration with there being no significant difference between each drug group and vehicle ($P>0.05$).

3.2.3. Joint inflammation

Subcutaneous dosing of 3 mg kg^{-1} morphine or 15 mg kg^{-1} celecoxib had no effect on FCA-induced joint swelling. However, prednisolone at 1, 3 and 10 mg kg^{-1} all significantly reduced the average ipsilateral knee joint diameter, ($P<0.05$, compared with vehicle) during the dosing period (data not shown).

3.2.4. Correlation of the PAM with weight distribution

In order to determine the strength of the correlation between the results obtained from the two readouts, a Spearman's linear regression analysis was carried out. The mean PAM ipsilateral LWT for each group was plotted against the mean ratio of weight distribution of the same group, with results from all experimental days included. A strong correlation between the

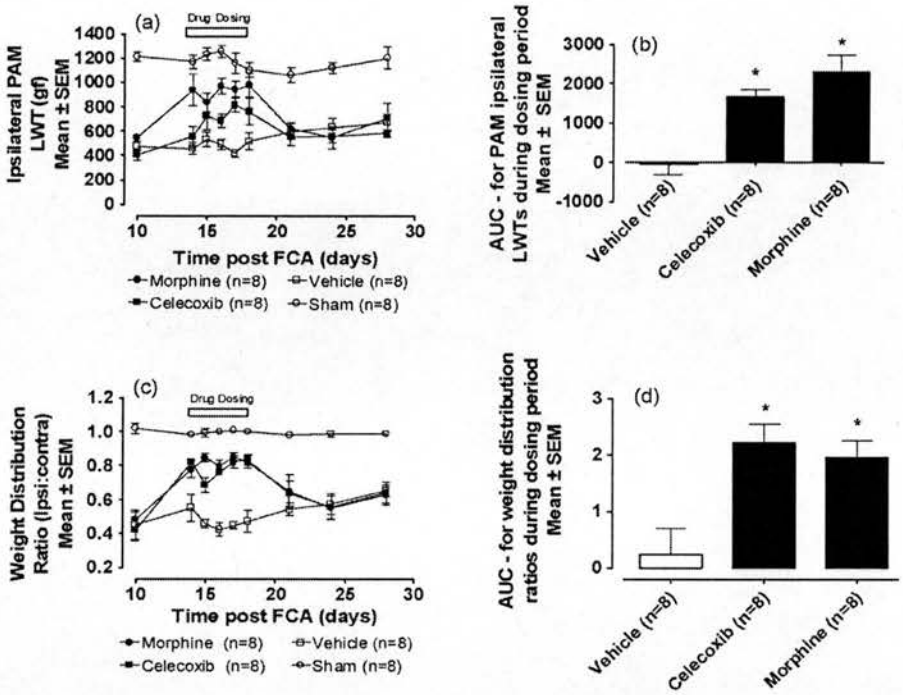


Fig. 4. The (a) ipsilateral PAM LWTs and (c) weight distribution ratios of vehicle-, morphine- and celecoxib-treated arthritic rats; and the corresponding graphs of AUC values for the two readouts during the dosing period (b and d). The drug-dosing period was between days 14 and 18 as indicated by the box on the graphs. PAM was able to detect the analgesic action of both drugs compared with vehicle. Statistical analysis was carried out to compare the AUC values for the drug-treated group to that of vehicle-treated animals using an ANOVA; *Statistical significance ($P < 0.05$).

results obtained from the two different readouts was observed (see Fig. 5a; Spearman $r = 0.91$; $P < 0.0001$).

3.2.5. *Power analysis of rat drug study*

The first power analysis performed (Fig. 5b) was calculated from the effect size equivalent to the observed difference between vehicle and the highest dose of prednisolone. Graphical results clearly show that in the structure of this experimental design, where each animal has three measurements taken for each readout, that a higher statistical power was achieved from the PAM readout compared with the weight distribution readout. In this experimental design if six animals per group were used for investigating a hypothesised drug effect equal to the observed effect, we could expect to achieve approximately 97% power using the PAM readout, but only 70% power with the weight distribution readout.

In a second power analysis (data not shown) results revealed that the statistical power could be greatly increased in the PAM readout, by increasing the number of measurements taken per animal from three to five, however increasing the number of animals used from 8 to 10 showed no real benefit. This was in contrast to the weight distribution readout, where increasing the number of animals from 8 to 10 greatly improved the power, but increasing the number of measurements showed no real benefit. This highlights the benefit of investigating the experimental design. For PAM we can actually increase the statistical power of an experiment by increasing the number of measurements while reducing the number of animals.

4. Discussion

This study has demonstrated the use of a novel behavioural tool for assessing joint pain in a rat model of experimental chronic joint inflammation. The results of these studies indicate that PAM provides a reliable, quantitative measurement of localised, FCA-induced mechanical hypersensitivity in the knee joint of rats. PAM was also able to detect the analgesic action of prednisolone, morphine and celecoxib. Furthermore, a strong correlation between the weight distribution readout and the PAM measurements were made, illustrating that PAM is a simple behavioural test that will be a valuable addition to current measures for assessing hypersensitivity in the joint.

Clinically, joint pain is assessed either subjectively using the Ritchie articular index or objectively using a dolorimeter (Langley et al., 1983; Ritchie et al., 1968). The Ritchie articular index allocates one of four grades of tenderness in patients' joints: "not tender" (0), "tender" (1), "tender and winced" (2) and "tender, winced and withdrew" (3). Previous studies carried out in this laboratory used a similar subjective scale of joint hypersensitivity (Gauldie et al., 2004). However, a quantitative measure, which mimics the pressure dolorimeter, would considerably improve assessments of experimental joint pain by providing an objective measure. The dolorimeter uses a gradually increasing force, applied in a perpendicular plane across the joint margin, to assess localised hypersensitivity of a human joint. The dolorimeter can be used on different joint types and has been adopted in clinical studies assessing osteoarthritis

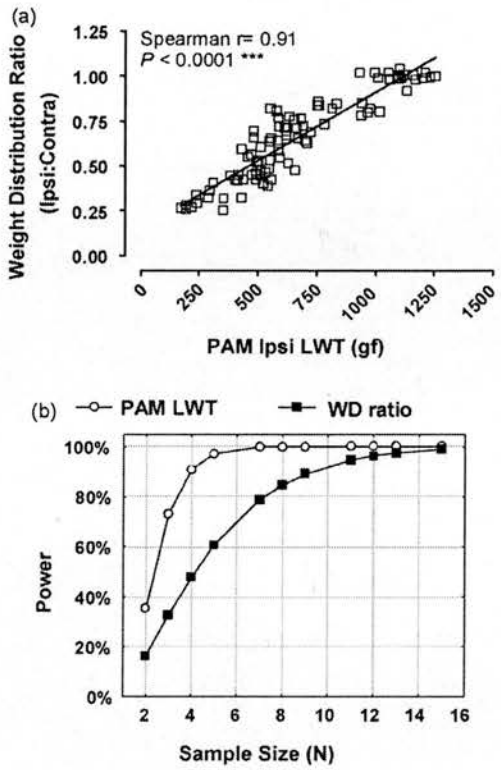


Fig. 5. (a) The correlation between the absolute ipsilateral LWTs (measured by PAM) and the ratio of the weight distribution between the hind limbs (measured by the incapacitance tester) in rats. There was a strong correlation between the two readouts (Spearman linear correlation factor, $r = 0.91$, $P < 0.0001$). (b) Analysis of the sample sizes required from the PAM and weight distribution (WD) readouts in triplicate measurements to reach certain statistical power levels. Results are calculated from an effect size equivalent to the observed difference between the vehicle group and the 10 mg kg^{-1} prednisolone group and results are based upon the observed variability in the study.

(Ottillinger et al., 2001) of the knee as well as the pain associated with fibromyalgia (Gracely et al., 2003). Currently, no objective measure of joint hypersensitivity is used to assess experimental arthritic joint pain in laboratory rodents. To that end, PAM was developed to align pre-clinical measures to those used clinically and help the translation of animal studies to human conditions.

Calibrated forceps have been used as a means of mechanical stimulation during electrophysiological recording (Gracely et al., 2003; Li and Neugebauer, 2004; Neugebauer and Li, 2002), or to measure knee joint or paw pain in experimental inflammation and primary hyperalgesia induced in deep tissues by inflammation (Han et al., 2005; Luis-Delgado et al., 2006; Yu et al., 2002). The latter involved direct application of the forceps to muscle or to the knee joint of rats (Cui et al., 1999; Skyba et al., 2005). However, although the technique is reported to be an easy to use, reliable method of assessing nociceptive withdrawal thresholds, they also show a steady increase in paw withdrawal threshold over a 9-day period that was highly correlated to an increase in weight gain over this time (Luis-Delgado et al., 2006). The authors suggest that the modification of pain sensitivity was due to increased size of the paw. This problem does not arise with PAM, as no change in pain threshold occurred over a 3-week period, despite the animals gaining weight in each study. In the pilot study a significant increase in LWT was noted in ipsilateral joints of naïve rats and contralateral joints of all animals. This increase was due to the absence of initial induction measurements prior to the start of the study. Subsequently, a pre-study behavioural assessment was carried out prior to studying the effect of chosen gold standard analgesics, removing this initial anomalous result.

The authors in these studies reported several problems with the forcep style of device, including variation caused by the operator due to finger placement on the forceps, and inaccurate values of withdrawal thresholds, as the calibrated reading given is a function of the force applied by the experimenter and the resistance offered by the joint. PAM is calibrated directly across the force transducer and directly records the precise force placed across the knee joint, up until the point of joint withdrawal. As PAM uses a single force transducer worn on the experimenters thumb, the amount of operator variation will only occur in the placement of the transducer on the knee joint of the animal being tested. However this is only a minor issue as the large surface areas of the PAM thumb attachment removes potential inaccuracy in focusing on an exact point on the joint, which happens in the case of small forcep tips. A further advantage of PAM is the ability to change the size of the pressure application surface and the force range of the transducer, allowing the device to be used in several species as well as the possibility of assessing other joints and paws.

In this study, PAM proved to be an easy to use device, which allowed rapid, reproducible measurements, using triplicate measurements at each time point taken. The absence of a significant difference between the ipsilateral and contralateral LWT in naïve animals, together with the low variability, indicated that PAM could use the contralateral joint as an internal control, an idea originally proposed by Randall and Selitto (1957).

Typical withdrawal thresholds for naive rats were approximately 1000 gf. These values are considerably higher than those seen in the paw using calibrated forceps or the Randall and Selitto device (Baamonde et al., 2004; Cook and Moore, 2006; Luis-Delgado et al., 2006; Randall and Selitto, 1957; Walker et al., 2003) which is probably due to the difference in the size and gross anatomy of the paw and the knee joint, in addition, the surface area over which the pressure is applied differs between the different devices. Pressure exerted onto the skin may activate nociceptive afferents in several tissues, depending on the surface area of the object used. Contact with a punctuate object such as a needle may exclusively activate nerve endings in the skin, in particular C fibres. Because deformation of the skin can be achieved with very small forces (Garell et al., 1996; Garnsworthy et al., 1988; Khalsa et al., 1997), these stimuli have little effect on afferents innervating deeper tissues. In contrast, a preferential activation of deep afferents is possible if pressure is exerted on a larger area of skin and the contact surface is rounded or padded (Treede et al., 2002). According to experiments using topical local anaesthesia, the contribution of cutaneous afferents to pain evoked by blunt pressure is minor (Kosek et al., 1995). This evidence adds support to the use of PAM as a measure of nociceptive activity in deeper tissues such as the joint itself,

rather than overlying skin. This is in contrast to von Frey hairs, which as a punctuate stimulus, activate skin afferents rather than those innervating the joint capsule and surrounding tissues.

Neugebauer and Li (2002) report that pressure stimuli >50 g mm^{-2} applied to the knee joint with calibrated forceps was noxious, as it consistently evoked hind limb withdrawal reflexes in awake rats. When values in our study were transformed into an approximate pressure the noxious LWT was ~20 gf mm^{-2}. However, the data from PAM is not routinely presented as a force per unit area (pressure), as it cannot be assumed that the entire surface of the disc is in contact with the joint, furthermore in inflamed joints the deformation of the joint onto the disc is different to that in naïve animals. PAM measurements are therefore more accurately expressed as a force, in grams.

PAM was able to detect FCA-induced hypersensitivity, observed as a decrease in LWTs of around 60% compared with the basal levels in normal joints. This hypersensitivity was time matched to the significant inflammation observed following FCA injection. The ability of PAM to detect FCA-evoked hypersensitivity provides an excellent experimental model in which to test the analgesic properties of novel compounds over a period of stable inflammation between days 14 and 18 post-FCA.

Prednisolone, a standard positive control in drug screening (Pyne et al., 2004), was studied over a 5-day period to check whether the drug would lead to a resolution of FCA-induced hypersensitivity as a correlate to the hyperalgesia we would expect to see in man. PAM detected prednisolone analgesia, over the 5 days of dosing, which was abolished after dosing ceased. PAM also showed significant reversal of evoked mechanical hypersensitivity in arthritic rats treated with the opioid morphine and the cyclooxygenase-2 inhibitor, celecoxib, which was comparable to that seen in the weight distribution readout. Dose-related reduction of hypersensitivity to prednisolone was not observed using PAM whereas a dose related analgesia was observed using the incapacitance tester. When using weight distribution, the global effects of each drug on the whole animal are determined, whereas the PAM device is measuring a localised effect at the knee joint. This may explain why the PAM results observed were not dose related, as the doses were sub-threshold for a dose–response curve to be observed as measured by the PAM device.

When results are examined strong correlations are seen between the two behavioural assessments. These results suggest that both techniques have the ability to detect a joint hypersensitivity, which can be attenuated using gold-standard analgesics. PAM has the added advantage of assessing the hypersensitivity of the joint at the site of the inflammation, in a similar fashion to the clinical dolorimeter (Langley et al., 1983).

The 3R's play a large part in the planning of *in vivo* studies, where the aim is always to reduce the number of animals used, or refine the techniques involved. In this study we have shown that PAM measurements can be collected alongside the usual weight distribution recordings, increasing the information from a single set of animals. The power analysis performed on this study has shown that PAM has a higher statistical power as a tool for measuring the efficacy of drugs used to reverse the FCA-induced hypersensitivity than the weight distribution readout. The power curves suggest that if PAM were used as a stand-alone readout in future studies then the number of animals per group could be reduced from eight to six, without compromising the statistical power of the study. Potentially PAM could be used to help reduce the number of animal's required in future joint hypersensitivity studies, but we are aware that these plots are based on the variability calculated from a single study. Replication of these results in further studies would increase the confidence in the conclusions drawn in this article.

In conclusion, the present study shows that PAM provides a novel, accurate behavioural tool for detecting a localised primary mechanical hypersensitivity in a rat model of chronic inflammatory joint pain. PAM is the first tool designed to measure both primary and secondary hypersensitivity objectively. It can be used in various experimental pain models and could be extended for use in other species. Results from these studies suggest PAM will aid the screening of novel analgesics designed to improve chronic inflammatory pain.

References

Ashburn MA, Staats PS. Management of chronic pain. Lancet 1999;353:1865–9.

Baamonde A, Lastra A, Fresno MF, Llames S, Meana A, Hidalgo A, et al. Implantation of tumoral XC cells induces chronic, endothelin-dependent, thermal hyperalgesia in mice. Cell Mol Neurobiol 2004;24:269–81.

Bedson J, Jordan K, Croft P. The prevalence and history of knee osteoarthritis in general practice: a case-control study. Fam Pract 2005;22:103–8.

Caldwell JR, Rapoport RJ, Davis JC, Offenberg HL, Marker HW, Roth SH, et al. Efficacy and safety of a once-daily morphine formulation in chronic, moderate-to-severe osteoarthritis pain: results from a randomized, placebo-controlled, double-blind trial and an open-label extension trial. J Pain Symptom Manage 2002;23:278–91.

Chillingworth NL, Donaldson LF. Characterisation of a Freund's complete adjuvant-induced model of chronic arthritis in mice. J Neurosci Methods 2003;128:45–52.

Clayton NM, Oakley I, Thompson S, Wheeldon A, Sargent B, Bountra C. Validation of the dual channel weight averager as an instrument for the measurement of clinically relevant pain. Br J Pharmacol 1997;120:219P.

Cook CD, Moore KI. Effects of sex, hindpaw injection site and stimulus modality on nociceptive sensitivity in arthritic rats. Physiol Behav 2006;87:552–62.

Cui JG, Meyerson BA, Linderoth B. Opposite effects of spinal cord stimulation in different phases of carrageenan-induced hyperalgesia. Eur J Pain 1999;3:365–74.

Donaldson LF, Seckl JR, McQueen DS. A discrete adjuvant-induced monoarthritis in the rat: effects of adjuvant dose. J Neurosci Methods 1993;49:5–10.

Garell PC, McGillis SL, Greenspan JD. Mechanical response properties of nociceptors innervating feline hairy skin. J Neurophysiol 1996;75:1177–89.

Garnsworthy RK, Gully RL, Kenins P, Mayfield RJ, Westerman RA. Identification of the physical stimulus and the neural basis of fabric-evoked prickle. J Neurophysiol 1988;59:1083–97.

Gauldie SD, McQueen DS, Clarke CJ, Chessell IP. A robust model of adjuvant-induced chronic unilateral arthritis in two mouse strains. J Neurosci Methods 2004;139:281–91.

Gracely RH, Grant MA, Giesecke T. Evoked pain measures in fibromyalgia. Best Pract Res Clin Rheumatol 2003;17:593–609.

Han JS, Bird GC, Li W, Jones J, Neugebauer V. Computerized analysis of audible and ultrasonic vocalizations of rats as a standardized measure of pain-related behavior. J Neurosci Methods 2005;141:261–9.

Khalsa PS, LaMotte RH, Grigg P. Tensile and compressive responses of nociceptors in rat hairy skin. J Neurophysiol 1997;78:492–505.

Kobayashi K, Imaizumi R, Sumichika H, Tanaka H, Goda M, Fukunari A, et al. Sodium iodoacetate-induced experimental osteoarthritis and associated pain model in rats. J Vet Med Sci 2003;65:1195–9.

Kosek E, Ekholm J, Hansson P. Increased pressure pain sensibility in fibromyalgia patients is located deep to the skin but not restricted to muscle tissue. Pain 1995;63:335–9.

Langley GB, Fowles M, Sheppeard H, Wigley RD. A simple pressure dolorimeter for the quantification of joint tenderness in inflammatory arthritis. Rheumatol Int 1983;3:109–12.

Le Bars D, Gozariu M, Cadden SW. Animal models of nociception. Pharmacol Rev 2001;53:597–652.

Li W, Neugebauer V. Block of NMDA and non-NMDA receptor activation results in reduced background and evoked activity of central amygdala neurons in a model of arthritic pain. Pain 2004;110:112–22.

Loeser JD, Melzack R. Pain: an overview. Lancet 1999;353:1607–9.

Luis-Delgado OE, Barrot M, Rodeau JL, Schott G, Benbouzid M, Poisbeau P, et al. Calibrated forceps: a sensitive and reliable tool for pain and analgesia studies. J Pain 2006;7:32–9.

Neugebauer V, Li W. Processing of nociceptive mechanical and thermal information in central amygdala neurons with knee-joint input. J Neurophysiol 2002;87:103–12.

Ottillinger B, Gomor B, Michel BA, Pavelka K, Beck W, Elsasser U. Efficacy and safety of eltenac gel in the treatment of knee osteoarthritis. Osteoarthr Cartilage 2001;9:273–80.

Pyne D, Ioannou Y, Mootoo R, Bhanji A. Intra-articular steroids in knee osteoarthritis: a comparative study of triamcinolone hexacetonide and methylprednisolone acetate. Clin Rheumatol 2004;23:116–20.

Randall LO, Selitto JJ. A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther 1957;111:409–19.

Ritchie DM, Boyle JA, McInnes JM, Jasani MK, Dalakos TG, Grieveson P, et al. Clinical studies with an articular index for the assessment of joint tenderness in patients with rheumatoid arthritis. Quart J Med 1968;37:393–406.

Schnitzer TJ, Weaver AL, Polis AB, Petruschke RA, Geba GP. Efficacy of rofecoxib, celecoxib, and acetaminophen in patients with osteoarthritis of the knee. A combined analysis of the VACT studies. J Rheumatol 2005;32:1093–105.

Skyba DA, Radhakrishnan R, Sluka KA. Characterization of a method for measuring primary hyperalgesia of deep somatic tissue. J Pain 2005;6:41–7.

Symmons DP. Looking back: rheumatoid arthritis—aetiology, occurrence and mortality. Rheumatology (Oxford) 2005;44(Suppl. 4):iv14–7.

Treede RD, Rolke R, Andrews K, Magerl W. Pain elicited by blunt pressure: neurobiological basis and clinical relevance. Pain 2002;98:235–40.

Walker SM, Meredith-Middleton J, Cooke-Yarborough C, Fitzgerald M. Neonatal inflammation and primary afferent terminal plasticity in the rat dorsal horn. Pain 2003;105:185–95.

Wilson AW, Medhurst SJ, Dixon CI, Bontoft NC, Winyard LA, Brackenborough KT, et al. An animal model of chronic inflammatory pain: pharmacological and temporal differentiation from acute models. Eur J Pain 2006;10:537–49.

Yu YC, Koo ST, Kim CH, Lyu Y, Grady JJ, Chung JM. Two variables that can be used as pain indices in experimental animal models of arthritis. J Neurosci Methods 2002;115:107–13.

Journal of Inflammation

BioMed Central

Research

Open Access

Demonstration of a novel technique to quantitatively assess inflammatory mediators and cells in rat knee joints

Nicola J Barton*[1], David A Stevens[2], Jane P Hughes[2], Adriano G Rossi[3], Iain P Chessell[2], Alison J Reeve[2] and Daniel S McQueen[1]

Address: [1]Division of Neuroscience, University of Edinburgh, Medical College, 1 George Sq, Edinburgh, EH8 9JZ, UK, [2]Neurology CEDD, GlaxoSmithKline R&D Ltd, Harlow, Essex CM19 5AW, UK and [3]MRC Centre for Inflammation Research, The Queens Medical Research Institute, University of Edinburgh, EH16 4TJ, UK

Email: Nicola J Barton* - N.J.Barton@sms.ed.ac.uk; David A Stevens - David.A.Stevens@gsk.com; Jane P Hughes - Jane.P.Hughes@gsk.com; Adriano G Rossi - Adriano.Rossi@ed.ac.uk; Iain P Chessell - Iain.P.Chessell@gsk.com; Alison J Reeve - Alison.J.Reeve@gsk.com; Daniel S McQueen - D.S.McQueen@ed.ac.uk

* Corresponding author

Published: 13 June 2007

Journal of Inflammation 2007, **4**:13 doi:10.1186/1476-9255-4-13

Received: 19 December 2006
Accepted: 13 June 2007

This article is available from: http://www.journal-inflammation.com/content/4/1/13

Abstract

Background: The inflammation that accompanies the pain and swelling associated with osteo- and rheumatoid arthritis is mediated by complex interactions of inflammatory mediators. Cytokines play a pivotal role in orchestrating many of these processes, including inflammatory cell recruitment, adhesion and activation. In addition, prostaglandins are secreted into the synovial cavity and are involved in perpetuation of local inflammation, vasodilatation and vasoconstriction, and also with bone resorption. Pre-clinical models have been developed in order to correlate to the human disease and principle among these is the adjuvant-induced arthritis model in the rat.

Methods: We have developed a technique to quantitatively assess the contents of synovial fluid samples from rat joints. Two needles joined together are inserted into the knee joint of anaesthetised rats and connected to a Watson-Marlow perfusion pump. Sterile saline is infused and withdrawn at 100 μl min^{-1} until a 250 μl sample is collected.

Results: Our results demonstrate up to 125 fold increases in synovial IL1α and IL1β concentrations, approximately 30 fold increases in levels of IL6 and IL10 and a 200–300 fold elevation in synovial concentrations of TNFα during FCA-induced experimental arthritis. Finally, this novel technique has demonstrated a dose-response relationship between FCA and the total cell counts of synovial perfusates.

Conclusion: In summary, this new technique provides a robust method for quantifying inflammatory mediators and cells from the synovial cavity itself, thereby detailing the inflammatory processes from within the capsule and excluding those processes occurring in other tissues surrounding the entire articulation.

1. Background

Inflammatory joint diseases such as rheumatoid arthritis (RA) are regulated by complex interactions involving many mediators, such as prostanoids and cytokines. The infiltration of cells into the synovial tissue and joint space is another key characteristic of synovitis, which combined with release of these mediators and degradative enzymes, eventually leads to cartilage and bone destruction (for reviews see [1]).

Measuring the levels of these mediators of inflammation in the synovial fluid from patients can provide information about the underlying pathophysiology of joint disease [2], for example the level of severity and current activity [3-5] as well as inter-individual variations in disease [6] and effectiveness of drug-treatments (for review see [7]). Furthermore changes occurring in the synovial fluid can be used as biomarkers of disease; this has already been demonstrated in RA patients with plasma levels of inflammatory proteins [8,9].

Human joint fluid samples have been taken and analysed for inflammatory mediator content from both healthy volunteers and patients with joint diseases. These studies revealed the importance of particular cytokines, including Tumour Necrosis Factor (TNF)α, Interleukin (IL) 1β, and IL6, which are now targets for disease-modifying anti-rheumatic drugs (DMARDs; for review see [10,11]). Furthermore increases in virtually all the prostanoids have been detected from these samples [12,13], but notably Prostaglandin E_2 (PGE_2), which has been associated with erosion of bone and cartilage in RA [14-17].

Although studies have investigated the fluid taken from joints, most research has focused on the inflammatory mediators within the synovial membrane, rather than those released into the intra-articular space. One reason for this is the technical difficulty of trying to assess cytokine levels in such a viscous material as synovial fluid. Several studies have assessed cytokine gene expression levels in the synovial membrane, rather than the actual protein content, both in human clinical samples [18,19] and in animal models of arthritis [20-22]. In addition, PGE synthase, the enzyme responsible for the conversion of cyclooxygenase-derived PGH_2 to PGE_2 has been detected in synovial tissues of patients with RA [23].

The early time course of release of key mediators cannot be determined using human synovial fluid samples, as patients rarely report to the clinic until the disease has progressed and is causing chronic pain and swelling [24]. Even then, repeated sampling from individuals is difficult, and most patients are prescribed drugs, to improve their symptoms and quality of life, which interfere with inflammatory regulatory processes and cytokine expression. Therefore by using animal models of disease, the early events of inflammation can be elucidated, and the effects of drugs on inflammatory markers can be measured under controlled conditions.

Rat adjuvant-induced unilateral arthritis is a well established RA disease model. [25-27] and use of this model has gone a long way in aiding the understanding of the time-course of the pathology in clinical RA. The model closely mimics the pathology of human RA, including histopathological changes, cell infiltration, hypersensitivity and swelling of the affected joint [28-30]. Previous studies in animal models of joint inflammation have investigated the time course of cytokine protein or gene expression using homogenates of whole rat joints or paws *post mortem* [20-22,31-33]. A major limitation of these studies is that such sampling always includes bone, synovial tissue, synovial fluid and surrounding muscles and connective tissue, which will not allow the origin of any analytes to be determined. Others have surgically dissected and lavaged knee joints in order to collect the synovial fluid from dead animals [34-36]. However, this does not allow for acute repeated sampling from the same animal over a period of up to a day to determine the affect of drugs on the levels of inflammatory mediators, or the acute effect of an inflammatory insult on inflammatory processes in the synovial cavity, a significant benefit of the perfusion method described here. A further study used an *in vivo* microdialysis procedure to determine the levels of inflammatory mediators in the synovial fluid of rats with adjuvant induced polyarthritis [37]. However, the apparatus used for this had limitations, for example the molecular weight cut-off of the microdialysis membrane was 50 kD, and therefore potentially underestimated the levels of IL1β in the joints. Furthermore, this limits the molecules that could be assessed by this method, which is in contrast to the present method, in which there is no limit to the size of molecules collected. The perfusion technique described in the present study also allows for the collection of cells from the joint space. As yet, no studies appear to have been carried out by perfusing saline through the intact joint space and collecting samples of cells and mediators from intact anaesthetised animals. The primary aim of this study was to develop a perfusion method to sample only the synovial fluid. A secondary aim was to study the effects of a joint insult on the intra-articular cytokine concentrations and cell infiltrate levels associated with adjuvant-induced arthritis in the joint space were also measured, as these are known key mediators in human RA conditions.

2. Methods

Experiments were performed in accordance with Home Office regulations and within UK animal welfare guidelines, and received Local Ethics Committee approval.

Male Wistar rats (Charles River, UK; initial weight ranges 240–290 g) were used. Rats were housed four to a cage in a 12-h light: dark environment and were given free access to standard animal feed and water for the duration of the study.

2.1 Arthritis induction

Briefly, rats (8) were transiently anaesthetised using 3% halothane in oxygen. The left knee was injected with 150 μl of Freund's Complete Adjuvant (FCA; 1 mg ml^{-1} Mycobacterium tuberculosis, Sigma, UK; i.art). A further 3 rats received a higher dose of FCA (500 μg), in order to assess the effect of adjuvant dose on inflammatory cell recruitment and mediator release into the joint space (100 μl; 5 mg ml-1 Mycobacterium tuberculosis, MAFF, UK; i.art). Only 3 rats were used for this part of the study, as it was designed as a pilot study to determine whether differences in the number of inflammatory cells and mediators present in the knee joint were evident between normal animals and those injected with the two doses of adjuvant using this new technique. The right joints were untreated. Animals were then allowed to recover from the anaesthesia.

2.2 Perfusion of joint space and analysis of samples

2.2.1. The perfusion needles

A needle perfusion system was constructed by binding a 25- and a 23-gauge needle together using epoxy putty, with the bevels of the needles positioned on the outside edges facing away from one another (see Figure 1). The tips of the needles were set 1–1.5 mm apart.

2.2.2. Perfusion of knee joints

Rats were anaesthetised with urethane (ethyl carbamate; 0.6 ml 100 g^{-1} body weight; 25% w v^{-1} solution; single i.p. injection). Once fully anaesthetised the animal was laid on its back on an automated heating blanket (Harvard Apparatus Limited, UK) and its core body temperature maintained at 37 °C via a thermistor probe positioned in the rectum.

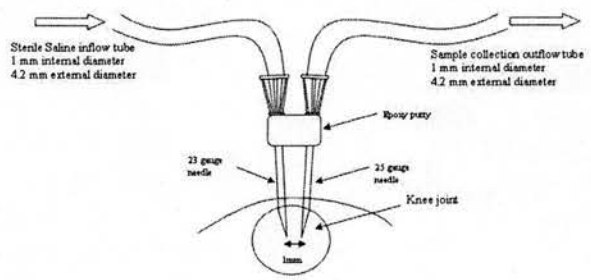


Figure 1
The perfusion needles and the perfusion system managing inflow and outflow from the knee joint space. A Watson-Marlow pump controlled the rate of saline infusion and sample extraction (100 μl min^{-1}) from the joint. After the knee was secured to prevent movement of the limb, needles were inserted into the knee joint through the patella tendon.

The limbs of the rat were flexed over a 20 ml glass vial, with the patella facing directly upwards for insertion of the perfusion needles, and the limb was secured in place with tape. The 23-gauge needle was connected to a Watson-Marlow roller pump via silicone rubber perfusion tubing (internal diameter 1 mm, external diameter 4.2 mm, Watson Marlow, UK). Sterile saline was infused at a constant rate of 100 μl min^{-1}. After infusion of 100 μl of vehicle (sterile saline), the outflow tubing was connected to the 25-gauge needle, to minimise pressure build-up within the joint space. Fluid was infused and withdrawn at a constant rate until a 250 μl basal sample was collected in a 1.5 ml centrifuge tube. Samples were immediately frozen at -20 °C.

2.2.3 Cytokine assay of joint samples

Luminex assay

Samples from the studies investigating the effects of anaesthetic on joint cytokine levels (n = 10) and the differences between normal (n = 10), high dose FCA-injected (n = 3) and low dose FCA- injected joints (n = 8) were analysed using a multi-cytokine bead array detection system capable of detecting rat IL1α, IL1β, IL2, IL4, IL6, IL10, Interferon (IFN) γ, Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and TNFα, according to the manufacturers instructions (Bio-Rad cytokine rat 9-plex, Biorad, USA). Briefly, a monoclonal antibody directed against the desired analyte was covalently coupled to dyed 5.5 μm polystyrene beads (2.5 × 10^6 beads ml^{-1} cytokine^{-1}). The conjugated beads were exposed to 50 μl of sample or standard solutions containing a known amount of cytokine, in a 96-well filter plate and incubated overnight at 4 °C, protected from light. After a series of washes and vacuum filtration to remove unbound protein, a biotinylated detection antibody specific for a different epitope on the analyte was added to the reaction. After incubation, the unbound antibody was removed; the reaction mixture was detected by the addition of streptavidin-phycoerythrin (streptavidin-PE), which binds to the biotinylated detection antibodies. Following a further series of washes and vacuum filtration, the beads were re-suspended in 200 μl 5% BSA in PBS; the plate was stored at 4 °C in the dark until analysis. The reaction mixture was read using a Luminex Data Collector in a Luminex 100 flow cytometer (Luminex, USA). The minimum detection limit of the assay was 2 pg ml^{-1} for each mediator measured. Any values lower than these levels were classed as 0 for the purposes of this study.

Luminex data analysis

Excel data files were generated containing individual bead numbers and the associated median fluorescence intensities. Standard curves were plotted to calculate the relative amount of each cytokine in samples, using the aliquoted serial dilutions of a positive control solution for calibration. Unknown sample cytokine concentrations were calculated from the curve.

ELISA assay

The levels of TNFα and ILβ in samples from studies investigating the effects of the needles (n = 6), and leakage of infusion from the joint cavity (n = 2) were measured using commercially available ELISA kits that specifically recognize the rat cytokines (BioSource International, Camarillo, USA) according to the manufacturer's instructions. Briefly, 100 μl aliquots of sample were pipetted into the wells of a microtiter plate pre-coated with an antibody specific for rat IL-1β or TNFα and incubated for 3 h at room temperature. After washing, a different biotinylated anti-rat IL-1β or TNFα antibody was added and incubated at ambient temperature for 1 h. Streptavidin-peroxidase was added and incubated for 30 min. After a third incubation and washing to remove all unbound enzyme, colour was developed by addition of stabilized chromogen (tetramethylbenzidine), a stop solution added and the intensity of the coloured product quantified spectrophotometrically at 450 nm. The minimum detection limit of the assay was 2 pg ml^{-1}.

2.3 Study design

2.3.1 Anaesthetic effects

In order to determine what effect anaesthetic agents had on inflammatory mediators in joints, control experiments were carried out. Firstly, five naive rats were anaesthetised with urethane (ethyl carbamate; 0.6 ml 100 g^{-1} body weight; 25% w v^{-1} solution; single i.p. injection), and five further rats with sodium pentobarbital (1 ml kg^{-1} body weight; 60 mg ml^{-1} solution; single i.p. injection maintained with i.v. 375 μl hr^{-1} 20 mg ml^{-1} solution of pentobarbital). No other procedures were carried out for 7 hours, at which point perfusion needles were inserted into both knee joints and a 250 μl sample collected. The sample was frozen immediately at -20°C, and later assayed using the Luminex assay.

2.3.2 Needle effects

In order to determine what effects inserting the perfusion needles had on synovial cytokine concentrations, an experiment was carried out in which six animals were anaesthetised with urethane (as described above), and the perfusion needles inserted into both knee joints and held in position for 7 hours, at which time a 250 μl sample was collected. The sample was frozen immediately at -20°C, and later assayed using an ELISA.

2.3.3 Perfusion effects on the concentration of analyte

Two naïve rats were anaesthetised with urethane (as described above) and a basal sample taken immediately. Then 1000 pg recombinant rat IL1β (Bioclone, USA) in 100 μl was infused over 1 min. A second sample was taken1 hour later; this was repeated hourly until 7 hours post-IL1β infusion. The samples were frozen and later assayed for IL1β content using an ELISA, to determine if the sample contained the same amount of IL1β that was initially infused.

2.3.4 Cytokine levels in normal and FCA-injected joints

Basal samples from ipsilateral and contralateral joints of 10 normal animals were compared with basal samples from 8 rats which had received i.art low dose FCA (150 μg) and 3 that were injected with i.art high dose FCA (500 μg) 14 days earlier. Samples (250 μl) were collected and frozen for later testing with the Luminex bead array.

2.3.5 Total cell counts

Joint perfusion samples were collected from ten naïve rat knee joints, eight 150 μg FCA-injected ipsilateral and contralateral joints and three 500 μg FCA-injected ipsilateral and contralateral joints. Undiluted samples were viewed by light microscopy in a haemocytometer. If red blood cells were present, or a high number of inflammatory cells, samples were diluted in saline, with added Zappoglobin, as per the manufacturer's instructions (1 drop per 20 ml).

2.4 Data Analysis

Data were collected and analysed using Microsoft Excel and Graphpad Prism software. Results are expressed as mean ± standard error of the mean (SEM) where appropriate.

Statistics

The Mann-Whitney U (non-parametric) test was used to analyse differences between groups, which were not normally distributed, or in which the sample size was small. To determine differences between the means of more than two groups a non-parametric one-way analysis of variance (Kruskal-Wallis) test was performed and a post-hoc test (Dunn's) undertaken if the test was significant. In all cases the null hypothesis was rejected at $P < 0.05$.

3. Results

3.1 Anaesthetic effects

Samples from naïve animals (n = 5) which received no treatment during 7 hours of urethane anaesthesia, showed a slight trend for increased levels of cytokines, but the increases were not statistically significant for IL1α, IL1β, IL2, IL4, IL6, IL10, GM-CSF, IFNγ, or TNFα compared with samples taken from rats immediately after administration of anaesthetic (n = 10; $P > 0.05$, Mann Whitney)

see Table 1. However, in contrast, animals anaesthetised with pentobarbital (n = 5), had significantly higher levels of GM-CSF and TNFα ($P < 0.05$, Mann Whitney) after 7 hours, in comparison with naïve joints, see Table 1.

3. 2 Needle effects

Samples taken from knee joints in which the perfusion needles had been in place for 7 hours while the animal was anaesthetised with urethane (n = 6) showed increased levels of TNFα, as measured by ELISA, but these were not statistically significant from basal samples from the same rats immediately after needle insertion ($P > 0.05$, Mann Whitney). IL1β levels in two joints increased to approximately 40 pg ml^{-1} over this time period, see Figure 2.

3.3 Perfusion effects on the concentration of analyte

Two joint perfusions were carried out to determine if any of the infused solution leaked from the joint space prior to withdrawal of samples. Recombinant rat IL1β (1000 pg), a cytokine known to be detectable by ELISA, was infused into the joint, along with saline, and samples were collected hourly. In both cases the full amount (1000 pg) administered was recovered in the first two samples. However, a greater amount of IL1β was recovered compared to the initial dose administered; Table 2 shows the results.

3.4 Levels of cytokines in normal and FCA-injected joints

Fourteen days after rats received 150 μg or 500 μg FCA i.art (n = 8 and 3 respectively), the ipsilateral joint contained significantly higher levels of IL1α, IL1β, IL6 and TNFα compared with samples from naïve joints (n = 10), as measured by the Luminex assay ($P < 0.05$, Two-way ANOVA; see Figure 3a). The contralateral joints of rats injected with 500 μg FCA also contained significantly higher levels of IL1α, IL1β, IL6 and TNFα ($P < 0.05$, Two-way ANOVA; see Figure 3b).

3.5 Total cell counts

Total inflammatory cell counts from normal animals (n = 5) and those injected with FCA (n = 8) 14 days prior to sampling are shown in Figure 4. Normal joints had no cells detectable, whereas all others samples had measurable levels. However, only the 500 μg FCA ipsilateral (n = 3) joints proved to have a significantly greater number of cells than normal joints ($4.8 \pm 0.06 \times 10^6$ cells ml^{-1}; $P < 0.05$, Mann Whitney). A dose-response relationship was demonstrated by the total cell count in both ipsilateral and contralateral joints.

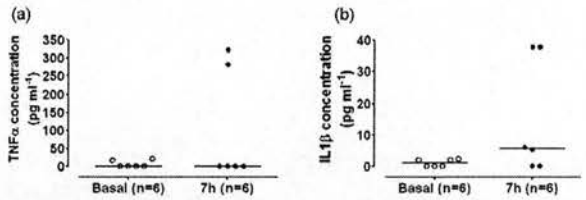


Figure 2

Levels of (a) TNFα and (b) IL1β from joints immediately after needle insertion (basal), and 7 hours later. Cytokines were assayed using an ELISA, and although there was an apparent increase in TNFα concentrations, to approximately 300 pg ml^{-1} in two samples, this was not statistically significant ($P > 0.05$, Mann Whitney). The horizontal lines on the graphs represent the median values in each group.

4. Discussion

The main aim of this study was to develop a method for sampling synovial fluid from the knee joint of anaesthetized rats. The technique was firstly validated by assessing whether any inflammatory response was evoked by the experimental set up, including the anaesthetic or the needles themselves; the efficiency of the system was investigated, *i.e.* whether any infused solution leaked from the joint space prior to sample extraction. Once the above factors had been assessed, they were taken into consideration when comparing samples from naïve and adjuvant-injected inflamed joints. Finally, the novel perfusion technique was used to quantify inflammatory cell numbers within the rat synovial cavity. This technique proved to be reliable and consistent when perfusing the joint cavity, and regular volumes of sample were easily collected. There were no problems with measuring protein content due to high sample viscosity, and this technique is therefore a valuable addition to protocols which use homogenates of entire joints to assess inflammatory mediator content.

Table 1: The effect of anaesthetic on basal levels of cytokines in the joint.

	IL1α	IL1β	IL2	IL4	IL6	IL10	GM-CSF	IFNγ	TNFα
Basal (n = 10) Mean ± SEM	0.8 ± 0.5	1.0 ± 1.0	0.5 ± 0.5	0.2 ± 0.2	2.5 ± 2.5	1.5 ± 1.0	0 ± 0	0.1 ± 0.1	0.2 ± 0.2
Urethane (n = 5) Mean ± SEM	2.6 ± 1.1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.0 ± 0.6	0.3 ± 0.3	36.6 ± 20.9
Pentobarbital (n = 5) Mean ± SEM	1.4 ± 0.7	0.2 ± 0.2	0.1 ± 0.1	0 ± 0	0 ± 0	6.2 ± 6.2	1.7 ± 0.4*	0.1 ± 0.1	44.2 ± 21.6*

Levels of nine cytokines in rats anaesthetised for 7 hours with either urethane or pentobarbital, in comparison with samples taken immediately after urethane anaesthesia (basal). Mann Whitney test were performed to determine differences between each anaesthetic and basal levels. Pentobarbital anaesthesia resulted in a significant elevation of GM-CSF and TNFα levels; statistical significance $P < 0.05$ indicated by *.

Table 2: Perfusion effects on the concentration of analyte.

	Animal 1		Animal 2	
	IL1β concentration (pg ml^{-1}; 250 μl)	**Amount of IL1β (pg)**	**IL1β concentration (pg ml^{-1}; 250 μl)**	**Amount of IL1β (pg)**
1 hour	2000	500	2000	500
2 hour	2000	500	2000	500
3 hour	200	50	356	89
4 hour	544	136	216	54
5 hour	350	87.5	210	52.5
6 hour	458	114.5	200	50
7 hour	318	79.5		
Total (pg)	**1467.5**		**1245.5**	

IL1β concentrations in each 250 μl sample collected, up to 7 hours post-infusion of IL1β (1000 pg). The amount of IL1β protein in each sample was calculated and summed, to show that little or no leakage from the joint space occurred. In fact, more IL1β was present than was injected, in both cases as a result of *de novo* release of endogenous IL1β protein.

It was established that the choice of anaesthetic may play a role in initiating an inflammatory response within the knee joints. Urethane, a hypnotic anaesthetic agent commonly used for laboratory animals, resulted in very little change in any of the mediators measured over a 7 hour period. In contrast, pentobarbital (pentobarbitone), a short-acting anaesthetic which must be maintained by i.v infusion, therefore requiring further surgical preparation of the animal, induced increases in GM-CSF and TNFα after continuous administration during the day, perhaps a result of the surgery of the implanted cannulae. It was therefore decided to use urethane for experiments, given that it provides an extended period of anaesthesia with minimal physiological changes [38], without the need for invasive surgical preparation. Furthermore, pentobarbital can cause respiratory depression in rats, whereas urethane causes minimal cardiopulmonary disturbances [38,39].

Once it was established that urethane anaesthesia had no adverse effects on the system, it was necessary to evaluate any inflammatory component as a result of the perfusion needles themselves, over a sustained time period of 7 hours. It was noted that a few rats developed increased TNFα or IL1β levels as a result of the needles being maintained within the joint. However, the change occurred in only 20% of animals, and was not significant; moreover,

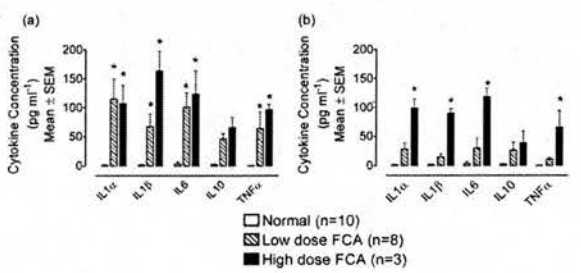


Figure 3
Levels of IL1α, IL1β, IL6, IL10 and TNFα in (a) ipsilateral and (b) contralateral joints of normal rats and those injected with low (150 μg; n = 8) and high (500 μg; n = 3) dose FCA 14 days earlier. There were negligible levels of any of the mediators measure in naïve joints (n = 10), but a significant increase in the expression of IL1α, IL1β, IL6 and TNFα was seen in all ipsilateral inflamed joints and in contralateral joints of rats injected with the high dose FCA ($P < 0.05$, Two-way ANOVA; compared with normal joints); statistical significance represented by *.

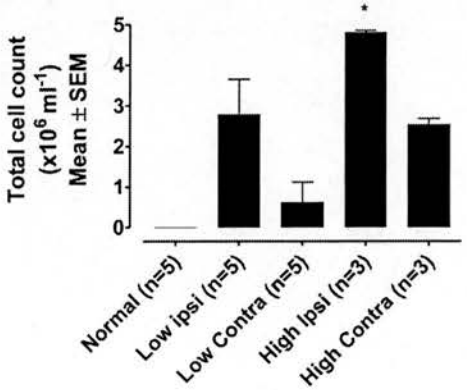


Figure 4
The effects of 150 μg (low dose; n = 5) and 500 μg (high dose; n = 3) FCA on total cell count from joint perfusates. Naïve joints contained no cells (0), whereas all other joints contained increased levels, although only high dose ipsilateral joints proved to have significantly raised levels ($P < 0.05$, Mann Whitney); statistical significance donated by *.

the increases in the two mediators did not occur in the same animals.

This study has demonstrated that very little, if any, solution infused into the joint is lost into the surrounding tissue, and can be recovered in full through the effusion tubes. This was confirmed by injection of Evans blue dye into the joint cavity and later dissection of the tissue (data not shown here). Furthermore, there was an increased quantity of IL1β detected in the perfusate collected. Although this study was not designed to show the effects of the protein on the joint, the 1 ng dose of IL1β administered resulted in *de novo* release of natural IL1β, as shown by the fact that elevated levels of IL1 were detected, in addition to the 1 ng dose.

Adjuvant-induced arthritis is a widely used model of inflammatory joint disease, and will be the primary subject of future studies applying this novel perfusion method. It was therefore important that samples collected in this way could detect differences between cytokine levels in naïve joints and FCA-treated joints. Levels of all cytokines measured in this study (IL1α, IL1β, IL6, IL10 and TNFα) showed dramatic increases 14 days after an initial inflammatory insult to the joint, including high and low doses of FCA. Furthermore, the contralateral joint of rats injected with the high dose of FCA also had higher levels of all cytokines measured, illustrating the contralateral effect also noted in the inflammatory cell count study. Finally, this study investigated the total number of white blood cells present in the joint washout samples. Not surprisingly it was observed that FCA-injected joints contained higher levels than normal rat knee joints, as previously shown [40]. However, of particular interest are the cell counts in contralateral, non-injected limbs. Contralateral effects arising from a unilateral insult is a well documented phenomenon. In general, contralateral changes in behaviour, magnitude of biochemical fluctuations or histopathological lesions are less than those observed on the ipsilateral side (for review see [41]). Total cell count data from this study are in agreement with this finding, and although the lower dose of FCA used here does not elicit behavioural signs of inflammation or hypersensitivity in the contralateral joint, there is evidence of infiltration of inflammatory cells.

5. Conclusion

In summary, we have demonstrated the use of a novel method for sampling synovial fluid and washing out the joint cavity to collect the "inflammatory soup", and have performed assays to measure levels of cytokines during adjuvant-induced arthritis. This method has the advantage of enabling the contents of synovial fluid to be investigated alone, without the contamination of the surrounding tissue. We have also revealed its value in measuring cellular components of inflammation. In conclusion, as this new method of joint perfusion uses anaesthetised animals, acute effects of anti-inflammatory drugs or novel compounds could be investigated, thus improving the knowledge of how novel drug targets are affecting the inflammatory process.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

NJB planned and carried out all *in vivo* studies, *in vitro* assays, *data* interpretation, statistical analysis and compilation of the manuscript. DAS and JPH assisted with the Luminex assay use and data collection, then read and edited the manuscript after completion. AGR assisted with the total inflammatory cell count studies and reviewed and edited the article. IPC, AJR and DSM contributed intellectually to the experimental designs, as well as to structural and editorial aspects of the paper. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank GlaxoSmithKline for funding these studies and my PhD studentship.

References

1. Sweeney SE, Firestein GS: **Rheumatoid arthritis: regulation of synovial inflammation.** *Int J Biochem Cell Biol* 2004, **36**:372-378.
2. Kubota E, Kubota T, Matsumoto J, Shibata T, Murakami KI: **Synovial fluid cytokines and proteinases as markers of temporomandibular joint disease.** *J Oral Maxillofac Surg* 1998, **56**:192-198.
3. Alstergren P, Ernberg M, Kvarnstrom M, Kopp S: **Interleukin-1beta in synovial fluid from the arthritic temporomandibular joint and its relation to pain, mobility, and anterior open bite.** *J Oral Maxillofac Surg* 1998, **56**:1059-65; discussion 1066.
4. Chang H, Israel H: **Analysis of inflammatory mediators in temporomandibular joint synovial fluid lavage samples of symptomatic patients and asymptomatic controls.** *J Oral Maxillofac Surg* 2005, **63**:761-765.
5. Rooney M, Symons JA, Duff GW: **Interleukin 1 beta in synovial fluid is related to local disease activity in rheumatoid arthritis.** *Rheumatol Int* 1990, **10**:217-219.
6. Ulfgren AK, Grondal L, Lindblad S, Khademi M, Johnell O, Klareskog L, Andersson U: **Interindividual and intra-articular variation of proinflammatory cytokines in patients with rheumatoid arthritis: potential implications for treatment.** *Ann Rheum Dis* 2000, **59**:439-447.
7. Barrera P, Boerbooms AM, van de Putte LB, van der Meer JW: **Effects of antirheumatic agents on cytokines.** *Semin Arthritis Rheum* 1996, **25**:234-253.
8. Eastgate JA, Symons JA, Wood NC, Grinlinton FM, di Giovine FS, Duff GW: **Correlation of plasma interleukin 1 levels with disease activity in rheumatoid arthritis.** 1988, **2**:706-709.
9. Houssiau FA, Devogelaer JP, Van Damme J, de Deuxchaisnes CN, Van Snick J: **Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides.** *Arthritis Rheum* 1988, **31**:784-788.
10. Christodoulou C, Choy EH: **Joint inflammation and cytokine inhibition in rheumatoid arthritis.** *Clin Exp Med* 2006, **6**:13-19.
11. Zwerina J, Redlich K, Schett G, Smolen JS: **Pathogenesis of rheumatoid arthritis: targeting cytokines.** *Ann N Y Acad Sci* 2005, **1051**:716-729.
12. Egg D: **Concentrations of prostaglandins D2, E2, F2 alpha, 6-keto-F1 alpha and thromboxane B2 in synovial fluid from**

patients with inflammatory joint disorders and osteoarthritis. *Z Rheumatol* 1984, **43**:89-96.
13. Trang LE, Granstrom E, Lovgren O: **Levels of prostaglandins F2 alpha and E2 and thromboxane B2 in joint fluid in rheumatoid arthritis.** *Scand J Rheumatol* 1977, **6**:151-154.
14. Dayer JM, Krane SM, Russell RG, Robinson DR: **Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells.** *Proc Natl Acad Sci U S A* 1976, **73**:945-949.
15. Fulkerson JP, Damiano P: **Effect of prostaglandin E2 on adult pig articular cartilage slices in culture.** *Clin Orthop Relat Res* 1983:266-269.
16. Robinson DR, Smith H, McGuire MB, Levine L: **Prostaglandin synthesis by rheumatoid synovium and its stimulation by colchicine.** *Prostaglandins* 1975, **10**:67-85.
17. Robinson DR, Tashjian AH Jr., Levine L: **Prostaglandin-stimulated bone resorption by rheumatoid synovia. A possible mechanism for bone destruction in rheumatoid arthritis.** *J Clin Invest* 1975, **56**:1181-1188.
18. Firestein GS, Alvaro-Gracia JM, Maki R: **Quantitative analysis of cytokine gene expression in rheumatoid arthritis.** *J Immunol* 1990, **144**:3347-3353.
19. Wagner S, Fritz P, Einsele H, Sell S, Saal JG: **Evaluation of synovial cytokine patterns in rheumatoid arthritis and osteoarthritis by quantitative reverse transcription polymerase chain reaction.** *Rheumatol Int* 1997, **16**:191-196.
20. Patten C, Bush K, Rioja I, Morgan R, Wooley P, Trill J, Life P: **Characterization of pristane-induced arthritis, a murine model of chronic disease: response to antirheumatic agents, expression of joint cytokines, and immunopathology.** *Arthritis Rheum* 2004, **50**:3334-3345.
21. Rioja I, Bush KA, Buckton JB, Dickson MC, Life PF: **Joint cytokine quantification in two rodent arthritis models: kinetics of expression, correlation of mRNA and protein levels and response to prednisolone treatment.** *Clin Exp Immunol* 2004, **137**:65-73.
22. Thornton S, Duwel LE, Boivin GP, Ma Y, Hirsch R: **Association of the course of collagen-induced arthritis with distinct patterns of cytokine and chemokine messenger RNA expression.** *Arthritis Rheum* 1999, **42**:1109-1118.
23. Westman M, Korotkova M, af Klint E, Stark A, Audoly LP, Klareskog L, Ulfgren AK, Jakobsson PJ: **Expression of microsomal prostaglandin E synthase 1 in rheumatoid arthritis synovium.** *Arthritis Rheum* 2004, **50**:1774-1780.
24. Cohen SB, Katsikis PD, Chu CQ, Thomssen H, Webb LM, Maini RN, Londei M, Feldmann M: **High level of interleukin-10 production by the activated T cell population within the rheumatoid synovial membrane.** *Arthritis Rheum* 1995, **38**:946-952.
25. Bileviciute I, Lundeberg T, Ekblom A, Theodorsson E: **Bilateral changes of substance P-, neurokinin A-, calcitonin gene-related peptide- and neuropeptide Y-like immunoreactivity in rat knee joint synovial fluid during acute monoarthritis.** *Neurosci Lett* 1993, **153**:37-40.
26. Billingham ME: **Mechanisms and Models of Rheumatoid Arthritis.** Edited by: Pettipher ER. London, Academic Press; 1995:389.
27. Mapp PI, Terenghi G, Walsh DA, Chen ST, Cruwys SC, Garrett N, Kidd BL, Polak JM, Blake DR: **Monoarthritis in the rat knee induces bilateral and time-dependent changes in substance P and calcitonin gene-related peptide immunoreactivity in the spinal cord.** *Neuroscience* 1993, **57**:1091-1096.
28. Donaldson LF, Seckl JR, McQueen DS: **A discrete adjuvant-induced monoarthritis in the rat: effects of adjuvant dose.** *J Neurosci Methods* 1993, **49**:5-10.
29. Pelegri C, Franch A, Castellote C, Castell M: **Immunohistochemical changes in synovial tissue during the course of adjuvant arthritis.** *J Rheumatol* 1995, **22**:124-132.
30. Wilson AW, Medhurst SJ, Dixon CI, Bontoft NC, Winyard LA, Brackenborough KT, Alba JD, Clarke CJ, Gunthorpe MJ, Hicks GA: **An animal model of chronic inflammatory pain: Pharmacological and temporal differentiation from acute models.** *European Journal of Pain* 2006, **10**:537-549.
31. Magari K, Miyata S, Nishigaki F, Ohkubo Y, Mutoh S, Goto T: **Differential effects of FK506 and methotrexate on inflammatory cytokine levels in rat adjuvant-induced arthritis.** *J Rheumatol* 2003, **30**:2193-2200.
32. Marinova-Mutafchieva L, Williams RO, Mason LJ, Mauri C, Feldmann M, Maini RN: **Dynamics of proinflammatory cytokine expression in the joints of mice with collagen-induced arthritis (CIA).** *Clin Exp Immunol* 1997, **107**:507-512.
33. Smith-Oliver T, Noel LS, Stimpson SS, Yarnall DP, Connolly KM: **Elevated levels of TNF in the joints of adjuvant arthritic rats.** *Cytokine* 1993, **5**:298-304.
34. Keeble JE Curtis, B, Mallaghan, FA & Brain, SD: **The role of sensory nerves in joint inflammation: studies using TRPV1 knockout mice.** *pA2 online* 2004, **2**:43P.
35. Singh HN, Blancuzzi V, Greenwood S, Skiles JW, O'Byrne EM: **Synovial fluid levels of tumor necrosis factor-alpha in the inflamed rat knee: modulation by dexamethasone and inhibitors of matrix metalloproteinase and phosphodiesterase.** *Inflamm Res* 1997, **46 Suppl 2**:S153-4.
36. Vale ML, Benevides VM, Sachs D, Brito GA, da Rocha FA, Poole S, Ferreira SH, Cunha FQ, Ribeiro RA: **Antihyperalgesic effect of pentoxifylline on experimental inflammatory pain.** *Br J Pharmacol* 2004, **143**:833-844.
37. Liu SH, Wong CS, Chang DM: **Increase Monocyte chemoattractant protein-1 in knee joints of rats with adjuvant-induced arthritis: in vivo microdialysis.** *The journal of rheumatology* 2005, **32**:2205-2211.
38. Sapru HN, Krieger AJ: **Cardiovascular and respiratory effects of some anesthetics in the decerebrate rat.** *Eur J Pharmacol* 1979, **53**:151-158.
39. Wixson SK, White WJ, Hughes HC Jr., Lang CM, Marshall WK: **The effects of pentobarbital, fentanyl-droperidol, ketamine-xylazine and ketamine-diazepam on arterial blood pH, blood gases, mean arterial blood pressure and heart rate in adult male rats.** *Lab Anim Sci* 1987, **37**:736-742.
40. Santos L, Tipping PG: **Attenuation of adjuvant arthritis in rats by treatment with oxygen radical scavengers.** *Immunol Cell Biol* 1994, **72**:406-414.
41. Shenker N, Haigh R, Roberts E, Mapp P, Harris N, Blake D: **A review of contralateral responses to a unilateral inflammatory lesion.** *Rheumatology (Oxford)* 2003, **42**:1279-1286.